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13. ABSTRACT (Maximum 200 Words)

In glandular organs, precancerous state precedes invasive carcinoma. In the breast this state is recognized as DCIS and consists of an epithelial cell proliferation confined by myoepithelial cells. Our laboratory has established cell lines/xenografts of myoepithelial cells. Our myoepithelial cell lines inhibit invasion and motility of breast carcinoma lines *in vitro* largely through maspin. The overall hypothesis of this proposal was how does myoepithelial maspin regulate breast (DCIS) carcinoma progression and can its detection in fine needle aspirates (FNA) and in ductal fluid abet diagnosis and screening? The first aim addressed the mechanism of maspin's inhibition of breast carcinoma invasion: We have shown that myoepithelial maspin does bind to plasma membranes of carcinoma cells and inhibit a pathway involved in cellular locomotion. Using differential display and microarray analysis we have further characterized the pathway(s) involved. The second aim utilized maspin antibodies on FNA to identify the myoepithelial component and we have further demonstrated with larger number of samples that this approach is successful. The third aim investigated the levels of maspin in nipple aspirates, in ductal lavage fluid and saliva. We have shown that maspin is both a tumor marker as well as a surrogate intermediate end point marker.

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INTRODUCTION

In many glandular organs, a precancerous state is thought to exist which precedes the development of frankly invasive carcinoma. In the breast this state is recognized as DCIS and consists of a proliferation of epithelial luminal cells confined by myoepithelial cells within the ductal system (1-7). CGH and LOH studies have failed to demonstrate a difference between DCIS and invasive breast carcinoma. Our laboratory has recently demonstrated that paracrine regulation of this transition by myoepithelial cells may be the main determinant of this important step in human breast carcinoma progression. Because of their close proximity to precancerous lesions, myoepithelial cells would be expected to exert important paracrine influences on these processes. Myoepithelial cells of the breast differ from ductal cells in many ways: they lack ER-α, and its downstream genes; they synthesize the adjacent basement membrane; they rarely proliferate or fully transform and give rise rarely only to low grade benign neoplasms. Myoepithelial cells are present around normal ducts and precancerous proliferations but are absent in invasive carcinoma. Our laboratory has established immortalized myoepithelial cell lines and xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3) and breast (HMS-4,5) (8-23). These cell lines and xenografts express identical myoepithelial markers as their *in situ* counterparts. Our myoepithelial cell lines and xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α-1 antitrypsin, an unidentified perhaps novel 31-33 kDa trypsin inhibitor (24), thrombospondin-1, soluble bFGF receptors, and maspin (25-34). The human myoepithelial cell lines, HMS-1, HMS-3, HMS-4, HMS-5 inhibit both ER-positive and ER-negative breast carcinoma invasion (down to 42%$\pm$7% of control) ($p<.05$) and in CM assays (down to 30%$\pm$8% of control) ($p<.01$).

The anti-invasive effects of HMS-1, HMS-3, HMS-4, HMS-5 and HMS-6 can be enhanced by phorbol 12-myristate 13-acetate (PMA) (down to 2%$\pm$1% of control) and abolished by dexamethasone (up to 95%$\pm$5% of control) ($p<.01$) Therefore with the appropriate pharmacological treatment, the myoepithelial cells do not only partially inhibit invasion --- they in fact near-totally eliminate it. PMA treatment causes an immediate and sustained release of maspin, a recently identified serpin. Immunoprecipitation of maspin from this CM nearly abolishes this anti-invasive effect. Adding purified native myoepithelial maspin to unconditioned media also results in a dramatic anti-invasive effect. Maspin exerts a similar inhibitory effect on breast carcinoma cell motility as noted by us using native maspin and others using both recombinant bacterial maspin and recombinant insect maspin(i) produced in Baculovirus-infected insect cells (35). Our findings suggest that myoepithelial-secreted maspin functions as a paracrine tumor suppressor, which may inhibit *in vivo* the progression of DCIS to invasive breast carcinoma.

The observation that myoepithelial cells express and secrete maspin has, in addition to these biological implications, important potential practical applications. Fine needle aspiration cytology of the breast is a safe noninvasive technique for diagnosing breast cancer that is being used with increasing frequency in older women and women with comorbid disease who would benefit from being spared a more invasive and anesthesia-requiring procedure such as lumpectomy. Presently however there is no way on FNA of distinguishing invasive breast cancer cells from DCIS cells. This is because both types of cells appear cytologically identical and on routine FNA there is no way to evaluate the cells in the anatomical context of the tissues. We feel however that if myoepithelial cells could be selectively identified on FNA then their presence would suggest the DCIS state since they would be expected to be aspirated along with the DCIS epithelial cells. Their absence on the smear would suggest that the

malignant cells which were present were derived from invasive carcinoma cells which are devoid of surrounding myoepithelial cells *in vivo*. Perhaps the absolute number or density of myoepithelial cells would also be discriminating factors. Our laboratory has shown that the immunocytochemical demonstration of maspin reliably distinguishes myoepithelial cells from all epithelial cells (normal, DCIS, and invasive carcinoma). Furthermore our laboratory has detected maspin in ductal fluid of the breast obtained by both nipple suction and selective breast ductal cannulation (36). This maspin in ductal fluid is produced by myoepithelial cells *in vivo* and reflects the integrity of the normal ductal lobular unit. Conceivably reduced levels of maspin in ductal fluid may reflect either structural or functional compromise of the myoepithelial layer and may be seen in high risk *v* normal women and/or ducts with abnormal microcalcifications, precancerous or invasive histopathology (37-40).

BODY (STATEMENT OF WORK)

1. To investigate the mechanism of maspin's inhibition of breast carcinoma cell invasion and motility. - Months 1-36

A. Maspin binding to breast carcinoma cells/plasma membranes. - Months 1-6

B. Identification and characterization of a maspin binding protein.- Months 6-18

C. Maspin activation of a breast carcinoma cellular pathway.- Months 18-36

We have confirmed both our initial and subsequent observations made during the first three years of funding and in the no cost extension period that purified myoepithelial maspin inactivates a cellular pathway involved in cellular locomotion. In addition we have utilized mRNA expression profiling of our myoepithelial tumors to demonstrate potential myoepithelial cellular pathways involved in maspin secretion and tumor suppression and have further identified some of the genes that are involved.

2. To utilize antibodies to maspin on fine needle aspirate (FNA) specimens to quantitate the myoepithelial component and determine if this determination discriminates between DCIS and invasive breast carcinoma. - Months 12-24

We initially made and then extended our observations to a much larger number of cases and further demonstrated that antibodies to maspin (polyclonal and monoclonal) are the best discriminator between myoepithelial cells and epithelial cells and they can be used to quantitate the myoepithelial component in FNA. We also conducted a prospective study using this same approach and extend this study in the no cost extension period. We plan to continue this prospective approach in a future grant application.

3. To investigate the levels of maspin in ductal fluid obtained by the nipple suction approach (in high risk *v* normal women) and in ductal fluid obtained by selective ductal cannulation and washings (ducts with and without microcalcifications from the same patient) and determine whether maspin levels abet screening and correlate with histological findings/ - Months 24-36

We had previously demonstrated that nipple aspirate fluids and ductal lavage fluids obtained by ductoscopy is rich in maspin as a surrogate intermediate end point marker that reflected the integrity of the ductal lobular unit. The methods used in these studies were contained in our publications (41,42). Ductal fluid contains numerous proteins in addition to maspin such as bFGF which may also be a marker for early cancerous changes. These points were made in another of our publications (43). Access to the ductal system of the breast and specifically targeting the myoepithelial-epithelial connection through gene therapy intraductal approaches may prove efficacious in early breast cancer chemoprevention and treatment, strategies initially claimed in our patent application (44) which has now become an official U.S. patent (45). In the latter parts of the sudy we focused more closely on this aim, expanding the number of cases and correlating the levels of maspin with the structural integrity of the ductal lobular unit. Our findings indicated that when the integrity of the ductal lobular unit is preserved the levels of maspin are not influenced by the degree of epithelial proliferation. The levels of other markers, eg. CEA, PSA, bFGF are however. When there is invasive cancer however and the myoepithelial layer is transgressed and / or destroyed, the level of maspin decreases or is absent. We have further confirmed this in studies conducted in the no cost extension period. We plan to continue these observations in a prospective randomized trial which will be the subject of a subsequent grant proposal.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that purified myoepithelial maspin binds to plasma membranes of carcinoma cells via a membrane receptor and both inactivates a cellular pathway involved in cellular locomotion and activates several other pathways determined by mRNA expression profiling. A series of myoepithelial cell lines and xenografts derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast and lung) exhibit common mRNA expression profiles indicative of a tumor suppressor phenotype. Previously established myoepithelial cell lines and xenografts (HMS-#; HMS-#X) were compared to non-myoepithelial breast carcinoma cells (MDA-MB-231, MDA-MB-468 and inflammatory breast carcinoma samples: IBCr, IBCw), a normal mammary epithelial cell line (HMEC) and individual cases of human breast cancer (zcBT#T) and matched normal human breast tissues (zcBT#N) (overall samples = 22). The global gene expression profile (22,000 genes) of these individual samples were examined using Affymetrix Microarray Gene Chips and subsequently analyzed with both Affymetrix and DChip algorithms. The myoepithelial cell lines / xenografts were distinct and very different from the non-myoepithelial breast carcinoma cells and the normal breast and breast tumor biopsies. 207 specifically selected genes represented a subset of genes that distinguished ($p<0.05$) all the myoepithelial cell lines / xenografts from all the other samples and which themselves exhibited hierarchical clustering. Further analysis of these genes revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors and proteinase inhibitors and decreased expression belonging to the classes of angiogenic factors and proteinases. Developmental genes were also differentially expressed (either over or underexpressed). These studies confirm our previous impression that human myoepithelial cells express a distinct tumor suppressor phenotype. We have further identified some of the key genes which are involved.

- We have further demonstrated using larger numbers of samples that antibodies to maspin (polyclonal and monoclonal) are the best discriminator between myoepithelial cells and epithelial cells and they can be used to quantitate the myoepithelial component. This can discriminate between pure DCIS and DCIS associated with invasion. Prospective studies testing this discrimination have been performed and will serve as a basis of a subsequent grant proposal.

- We have demonstrated that nipple aspirate fluids and ductal lavage fluids obtained by ductoscopy is rich in maspin as a surrogate intermediate end point marker that reflects the integrity of the ductal lobular unit. Ductal fluid contains numerous proteins in addition to maspin such as bFGF which may also be a marker for early cancerous changes. Access to the ductal system of the breast and specifically targeting the myoepithelial-epithelial connection through gene therapy intraductal approaches may prove efficacious in early breast cancer chemoprevention and treatment, strategies claimed in our patent application. We have further confirmed these points by additional studies conducted in the latter aspects of the study. Our findings indicated that when the integrity of the ductal lobular unit is preserved the levels of maspin are not influenced by the degree of epithelial proliferation. The levels of other markers, eg. CEA, PSA, bFGF are however. When there is invasive cancer however and the myoepithelial layer is transgressed and / or destroyed, the level of maspin decreases or is absent. We plan to continue these observations in a prospective randomized trial which will be the subject of a subsequent grant proposal.

REPORTABLE OUTCOMES

PUBLICATIONS

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8. **Barsky SH.** Myoepithelial mRNA expression profiling reveals a common tumor suppressor phenotype. *Experimental and Molecular Pathology* 74: 113-122, 2003.

9. Love SM and **Barsky SH**. Anatomy of the nipple and breast ducts revisited. *Cancer*, 2004, in press

PATENTS AND LICENSES

1. **Barsky SH** and Alpaugh ML. Compositions and methods for intraductal gene therapy. *U.S. Patent* 6,514,695; February 4, 2003

2. Love SM and **Barsky SH**. Methods for performing medical procedures within a breast duct. *U.S. Patent Application Serial No.:* 10/099,439, filed 10/31/02.

3. Love SM and **Barsky SH**. Methods for obtaining fluid and cellular material from a breast duct. *U.S. Patent Application Serial No.:* 10/175,022, filed 06/19/02.

BOOK CHAPTERS

1. **Barsky SH**, Kedeshian P and Alpaugh ML. Maspin and myoepithelial cells. Maspin edited by M.J.C. Hendrix. Landes Bioscience, Georgetown, Texas, 2002.

2. **Barsky SH** and Alpaugh ML. Myoepithelium: Methods of culture and study. Culture of Human Tumor Cells, edited R. Ian Freshney, John Wiley & Sons, Inc., New York, NY, 2002, in press

CONCLUSIONS

1. Mechanism of Maspin Action

Maspin activation / inactivation of a cellular pathway. The first question that we addressed was whether maspin exhibits specific, saturable, reversible, and displaceable binding to the surface of breast carcinoma cells in a manner of a ligand-receptor interaction and it does. The mechanism of maspin's effects on invasion and motility inhibition are still unknown. Our studies have shown that in myoepithelial cells it is *secreted* in large amounts. We have been able to purify native maspin to homogeneity. We have obtained *r*maspin from Dr. Zhang (Baylor). Both *r*maspins (bacterial and insect) and native maspin derived from myoepithelial cells have been iodinated and incubated with first intact MDA-231, MDA-468, MCF-7, T47D cells and then with their plasma membrane fractions. Excess unlabelled ligand was added and specific binding was determined with Scatchard analysis to calculate the Kd of binding and the # of binding sites/cell or /membrane protein. Specific, displaceable binding indicated a binding protein (receptor). The cell line with the highest maspin binding was used as source to isolate a maspin receptor. In year 02 we carried this approach further by identification and characterization of a maspin binding protein. Two approaches were used: Maspin was crosslinked to Sepharose 4B and an affinity column was made. The plasma membrane preparation from the breast carcinoma cell line exhibiting the highest maspin binding was extracted, iodinated by the lactoperoxidase method and added to the maspin affinity column. Controls included BSA cross-linked to Sepharose 4B or cross-linked Sepharose4B alone. The bound fraction was eluted with 0.2 M glycine HCl pH 3.5, immediately neutralized with 1.0M Tris/saline, lyophilized and run on a gel. Autoradiograms were used to identify a specific binding protein. Since maspin is a serpin, candidate receptor molecules included membrane associated proteinases such as the uPA/uPAR complex or MT-MMPs. Since maspin, in addition to inhibiting invasion also inhibits cell motility (which in itself could explain its inhibition of invasion), inhibition by binding to the handful of known motility-stimulating ligand-receptor complexes such as scatter factor/hepatocyte growth factor-c-met receptor, autocrine motility factor-receptor, autoxin-receptor, bFGF and its receptor, interleukin 6 and its receptor, integrins, and E cadherin was investigated by doing a simultaneous Western blot on the eluted material with antibodies to these different molecules. Since another possibility was that maspin was directly binding a negative regulator of cell motility rather than inhibiting a positive regulator, TGFβ-receptor and retinoic acid-receptor complexes, known negative regulators of cell motility were investigated by Western blotting. Recently investigators have shown that maspin binds to single stranded tissue plasminogen activator (ss t-PA); this molecule which is secreted could be a target for maspin action. However in the vast majority of the carcinoma and melanoma lines we studied where maspin exerted a pronounced suppressive effect on both motility and invasion, no ss t-PA was detected in these lines; hence maspin must be acting on a different target. Since both invasion and motility involve complex intracellular pathways, we hypothesize that maspin triggered a signal transduction pathway leading to inhibition of invasion/motility. For that reason we investigated by differential display and microarray analysis various genes whose expression levels were altered (up or down) by the actions of maspin. Computational analysis of these genes identified several candidate pathways of action. We have further analyzed this microarray analysis (expression profiling) in the latter aspects of the study.

The human myoepithelial cell lines / xenografts subjected to the present microarray analysis exhibited a unique gross and characteristic phase contrast appearance. Ultrastructural studies of the xenografts confirmed the presence of abundant extracellular matrix devoid of murine stromal cells,

inflammatory cells and endothelial cells (angiogenesis). These findings suggested that microarray analysis would reflect only human gene expression. Karyotype analysis of the transformed myoepithelial cell lines revealed only minimal deviations from a normal karyotype suggesting that they would serve as a suitable normal myoepithelial cell surrogate. Furthermore the same gene products could be detected within the transformed myoepithelial cells of the xenografts as within normal myoepithelial cells *in situ*. These findings suggested that subsequent microarray analysis would reflect the normal myoepithelial cell phenotype.

These myoepithelial cell lines / xenografts which had been derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast and lung) exhibited a common mRNA expression profile which was indicative of a tumor suppressor phenotype. With hierarchical clustering, a significantly distinct grouping of the myoepithelial cell / xenograft samples were observed based on an analysis of 3200 genes. With a subsequent analysis based on a subset of 207 genes ($p<0.05$) even tighter grouping of the myoepithelial samples was observed. Within this subset of 207 genes was an excess of developmental genes, both over and underexpressed. Additionally filtered genes, hierarchically clustered and rank ordered, exhibited a 3 fold differential (either over or underexpressed) between the myoepithelial samples and the non-myoepithelial samples. Analysis of these clusters revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors and proteinase inhibitors and decreased expression of genes belonging to the classes of angiogenic factors and proteinases. Numerous expressed sequence tags also clustered within each of the aforementioned classes. Northern blot, Western blot and functional assays, eg. zymograms confirmed the increased / decreased expression of selected genes noted on microarray analysis. We have begun to identify the specific genes which are involved.

2. Maspin in FNA

We have utilized maspin antibodies on FNA specimens to quantitate the myoepithelial component and determine if this determination discriminates between DCIS and invasive breast carcinoma. Polyclonal (rabbit) and monoclonal (murine) antibodies to maspin have been used (Pharmingen, San Diego, CA) according to standard immunocytochemical cytological protocols. We had great success with this approach in year 01 and expanded this to a much larger number of cases in year 02. We chose cases known by subsequent biopsy to be either pure DCIS or predominantly invasive carcinoma (most invasive breast cancers have at least some DCIS component). Without knowing the surgical pathology we determined first whether there were any myoepithelial cells present on the FNA and we then determined their number by assessing them by maspin positive immunostaining. We determined both the absolute number of myoepithelial cells and the ratio of myoepithelial cells to epithelial cells (the immunopositive to immunonegative ratio) and grouped the cases where there were no myoepithelial cells and the cases where there were some myoepithelial cells and determined the mean number $\pm$ standard deviation in this second group. We then subdivides these results into cases of known DCIS and cases of predominantly invasive cancer and did a t test on the results to see if DCIS and invasive breast cancer differed in their average number of myoepithelial cells present on FNA. Analyzing the results in this manner assumed and confirmed a Gaussian distribution for the number of myoepithelial cells in both groups. Based on the differences in the mean values and/or the slope of this curve we were able to perform power calculations to determine the number of cases needed to achieve statistical significance. Basically the steeper the slope of this curve the more

discriminating the myoepithelial measurement was and the fewer the number of cases needed. Based also on the differences in the mean values (how many standard deviations apart) of myoepithelial cell numbers or myoepithelial/epithelial ratios between our two groups, DCIS and invasive carcinoma, we were able also to perform power calculations and determine the number of cases needed. Furthermore we have used antibodies to smooth muscle actin, S100 and CALLA, all of which are fairly myoepithelial specific to compare with our maspin results. All of the latter antibodies recognized structural myoepithelial components and as such quantitate myoepithelial cell number. Maspin, on the other hand, not only identified myoepithelial cell number but provided an index of myoepithelial cell function---after all maspin is a serpin and a paracrine tumor suppressor. Therefore the use of maspin in this setting was more informative. In year 03 and in the no cost extension period we further expanded on the number of case and began prospective studies.

3. Maspin in Ductal Fluid

Maspin in ductal fluid was measured by Western blot with total protein normalization. Human subjects approval for the collection of ductal fluid through select duct cannulation was obtained.. Small aliquots of this collected ductal fluid (50 patients) was used for the present studies. Nipple aspirates were collected by nipple suction (Sartorius/Petrakis). Analysis of nipple aspirates revealed the pooled contributions of all or most of the ducts where selective ductal cannulation allowed for a discrimination of one duct from another (eg, a duct with DCIS or microcalcifications *v* a normal duct. Our findings indicated that maspin was present reflecting the integrity of the ductal-lobular unit. We also demonstrated maspin in saliva, a fluid which reflects the abundance of myoepithelial cells in the salivary glands (See publication in Appendix).

In year 02, ductal fluid was begun to be collected following cannulation and washing of selected ducts in patients with microcalcifications on screening mammography who are about to undergo either excisional or core biopsy. In year 03, we will expand this to a larger number of cases. Paired comparisons of maspin levels in ductal fluid obtained from ducts harboring microcalcifications or DCIS and normal ducts from the same patients have and will continue to be made. Maspin levels will be correlated with the histopathology surrounding the microcalcifications. We have shown that some of these patients exhibit normal ductal histopathology surrounding their microcalcifications, some exhibit proliferations like hyperplasia, adenosis, ADH, and DCIS and still others invasive carcinoma. The screening value of maspin levels in all of these patients is emerging. Our approach to the evaluation of maspin values and to their discriminatory ability among the different groups has been similar to that given above for the analysis of myoepithelial cell number. Measurements of *myoepithelial* maspin in ductal fluid will continue to be compared to levels of a breast *epithelial* cell marker such as carcinoembryonic antigen (CEA) or bFGF. We, in fact, have recently found that bFGF is elevated in ductal fluid of certain patients. Hence the maspin/CEA or maspin/bFGF ratio might be predictive of risk with increased maspin/CEA or increased maspin/bFGF correlating with normalcy and decreased maspin/CEA or decreased maspin/bFGF correlating with either high risk, microcalcifications and/or precancerous histopathology.

In year 03 we focused more closely on this aim, expanding the number of cases and correlating the levels of maspin with the structural integrity of the ductal lobular unit. Our findings indicated that when the integrity of the ductal lobular unit is preserved the levels of maspin are not influenced by the degree of epithelial proliferation. The levels of other markers, eg. CEA, PSA, bFGF are however. When

there is invasive cancer however and the myoepithelial layer is transgressed and / or destroyed, the level of maspin decreases or is absent. We continued these observations in the no cost extension period and are comtemplating a prospective randomized trial which will be the subject of a subsequent grant proposal.

PERSONNEL

The following individuals received salary support from this grant:

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APPENDIX

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Tamoxifen enhances myoepithelial cell suppression of human breast carcinoma progression in vitro by two different effector mechanisms

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Abstract

Our previous studies have indicated that myoepithelial cells surrounding ductal and acinar epithelium of glandular organs, such as the breast, exert multiple paracrine suppressive effects on incipient and developing cancers that arise from this epithelium. Myoepithelial cells and derived cell lines (HMS 1–6) exert these effects through the secretion of a number of different effector molecules that exert anti-invasive, anti-proliferative, and anti-angiogenic activities. Since previous basic and clinical studies have examined the role of estrogen agonists and antagonists on human breast cancer cells and because issues of hormone replacement therapy (HRT) and tamoxifen chemoprevention are such timely issues in breast cancer, we wondered whether or not hormonal manipulations might affect myoepithelial cells in vitro as far as their paracrine suppressive activities on breast cancer were concerned. The present in vitro study demonstrates that treatment of myoepithelial cells with tamoxifen but not 17β-estradiol increases both maspin secretion and invasion-blocking ability. Furthermore tamoxifen but not 17β-estradiol increases inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by myoepithelial cells when they are co-cultured with conditioned media from or breast carcinoma cells directly. This increased myoepithelial NO exerts both autocrine and paracrine antiproliferative effects which can be blocked by inhibition of iNOS. 17β-Estradiol, however, competes with all of these suppressive effects of tamoxifen suggesting that the mechanism of tamoxifen action is estrogen receptor mediated. Myoepithelial cells lack ER-α but express ER-β. Tamoxifen, but not 17β-estradiol, increases AP-1 CAT but not ERE-CAT activity. Again, 17β-estradiol competes with the transcription-activating effects of tamoxifen. These experiments collectively suggest that the actions of tamoxifen on the increased secretion of maspin and increased production of NO by myoepithelial cells are mediated through ER-β and the transcription-activation of an ER-dependent AP-1 response element.

Keywords: Myoepithelial cell; ER-β; Tumor suppression; Tamoxifen; Maspin; Nitric oxide

1. Introduction

Our previous studies have indicated that myoepithelial cells surrounding ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers that arise from this epithelium [1–3]. This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the evolving cancer exists for a number of years only as an in situ

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lesion confined within the ductal system [4]. This in situ lesion is commonly termed ductal carcinoma in situ or DCIS. Due to their close proximity, myoepithelial cells would be anticipated to exert important paracrine influences on both normal, precancerous, and cancerous epithelial cells. Myoepithelial cells of the breast differ from luminal ductal and acinar epithelial cells in many ways: they lie next to the basement membrane and contribute to the synthesis of that structure; they rarely transform or proliferate and when they do give rise to only low-grade benign neoplasms [5,6]. Myoepithelial cells, in a sense, can be regarded then as both autocrine as well as paracrine tumor suppressors. Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5) and bronchus (HMS-6) (with their respective xenografts designated as HMS-#X) [1–3,5,6]. These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells in situ and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells in situ constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α-1 antitrypsin, an unidentified 31–33 kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin [1–3]. Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion [1,3] and endothelial cell migration and proliferation (angiogenesis) in vitro [7]. Our myoepithelial cell lines also inhibit breast carcinoma proliferation in vitro through an induction of breast carcinoma cell G_2/M arrest and apoptosis [3], the latter phenomenon of which also occurs in situ within DCIS [8]. On the basis of our immunoprecipitation studies, secreted myoepithelial cell maspin is a major effector molecule that inhibits invasion but not proliferation [3]. Candidate myoepithelial molecules that mediate antiproliferative and antiangiogenic effects on carcinoma cells and endothelial cells respectively include nitric oxide (NO) and thrombospondin-1 [4].

The effects of hormone replacement therapy (HRT) and tamoxifen chemoprevention are timely issues for patients at risk for developing breast cancer but previous basic and clinical studies examining the effects of these hormones in human breast cancer have focused mainly on their direct actions on carcinoma cells themselves. For this reason, we wondered whether these hormones could conceivably affect breast carcinoma cells indirectly by their actions on myoepithelial cells. In the present study we specifically examined the hormonal regulation of the tumor suppressor phenotype of myoepithelial cells in vitro by focusing on two myoepithelial-associated effector molecules: maspin and NO.

2. Materials and methods

2.1. Reagents

Tamoxifen, 17β-estradiol, PMA (phorbol 12-myristate 13-acetate), dexamethasone, and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium (MEM), keratinocyte serum-free medium (KSFM) and fetal bovine serum (FBS) were obtained from Life Technologies Inc./Gibco (Grand Island, NY). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

2.2. Cell lines

The ER-positive (MCF-7 and T-47D) and ER-negative (MDA-MB-231 and MDA-MB-468) cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD). MCF-7, T-47D, MDA-MB-231, and MDA-MB-468 cells were maintained in Eagle MEM with 10% FBS and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Human myoepithelial cell lines (HMS-1, HMS-3, HMS-4, HMS-5, and HMS-6), established in our laboratory [1–3,5,6], were maintained in KSFM with epidermal growth factor and bovine pituitary extract supplements. All cells were cultured as monolayers in 95% air/5% CO_2. Conditioned media (CM) was prepared and concentrated as previous described [1].

2.3. In vitro assays of invasion

Invasion experiments were conducted with an invasion chamber utilizing Matrigel from Collaborative

Biomedical Products (Bedford, MA) as described in previous studies [1], using highly invasive breast carcinoma cell lines. The Matrigel invasion experiments were carried out according to standard protocols using an intervening layer of HMS-1 cells or concentrated CM from HMS-1 cells [1,9]. Our previous studies had shown that CM from HMS-1 cells had to be concentrated at least 25-fold for invasion-inhibitory activity to be observed [1]. Treatment of HMS-1 cells was carried out for 24 h with the following agents: PMA (5 μM), dexamethasone (0.25 μM), tamoxifen (10^{-8}, 10^{-7}, or 10^{-6} M) and 17β-estradiol (10^{-8},10^{-7}, or 10^{-6} M). For competition experiments, the dose of 17β-estradiol was increased to 10^{-5} M. Other myoepithelial cell lines were studied in similar fashion.

2.4. Growth experiments

HMS-1 cells were plated in 24-well plates at an initial cell concentration of 1×10^4 cells per well in supplemented KSFM, treated with various concentrations and combinations of tamoxifen and 17β-estradiol, and counted after 3, 6, and 9 days. Other myoepithelial cell lines were studied in similar fashion. The cells were also assayed for apoptosis by the ApopTag detection system (Oncor, Gaithersburg, MD) as performed in previous studies [3]. The effects of inhibition of inducible nitric oxide synthase (iNOS) by hydrocortisone (5 μM) were also studied in these settings.

2.5. Northern, Western, and immunoprecipitation studies

Total RNA was extracted, 20 μg of total RNA was loaded per lane in 1.2% agarose gels, and Northern blot analyses were performed as previously described [1]. The full-length human ER-α cDNA probe was provided Dr P. Chambon (IGBMC, France); the full-length human iNOS cDNA probe was provided by Dr Louis Ignarro (UCLA, Los Angeles, CA); a maspin cDNA probe was provided by Dr Ruth Sager (Dana-Farber Cancer Institute, Boston, MA). Probes were labeled according to the random primer method of Feinberg and Vogelstein [10]. Normalization was achieved with a 36B4 cDNA probe (ATCC). Scion Image software was used for densitometric analysis of the bands. Western blots were performed as previously described [1] using a mouse monoclonal antibody to maspin (PharMingen, San Diego, CA) with the manufacturer's recommended dilutions and a 1:50 000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL).

Concentrated HMS-1 CM (25×) was immunoprecipitated by using anti-maspin antibody (PharMingen) at various dilutions (1:100–1:2000) followed by immunobeads (Bio-Rad). Following centrifugation, the supernatants were harvested, filtered through a 0.2-μm filter, and saved for invasion experiments and Western analysis. The immunoprecipitates were analyzed by Western blot. Control antibodies used included mouse monoclonals anti-human PAI-1 and PAI-2 (American Diagnostica, Greenwich, CT).

2.6. Determination of nitric oxide (NO) and iNOS expression

HMS-1 cells were cultured both in the presence and absence of MDA-MB-231 cells and in the presence and absence of MDA-MB-231 CM (25 ×). The cells received either added tamoxifen (10^{-7} M), 17β-estradiol (10^{-7} M) or various concentrations and combinations. After various time periods (0–24 h), the HMS-1 cells in the CM experiments were harvested for Northern blot analysis. In both the co-culture experiments and the CM experiments, the supernatants were analyzed after 24–72 h for nitrite content. NO, quantified by the accumulation of nitrite (as a stable end product) in the supernatants, was determined by a microplate assay method [11]. Briefly, 100 μl samples were removed from the conditioned supernatants and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-1-naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve and expressed in micromolar amounts. Values were normalized for total cellular protein and NO expressed in arbitrary units. In the co-culture experiments, the cells were also assayed for apoptosis by the ApopTag detection system (Oncor, Gaithersburg, MD) as performed in previous studies [3]. The effects

of inhibition of iNOS by hydrocortisone (5 μM) were also studied in these settings.

2.7. Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA (1 μg per reaction), denatured at 65°C for 5 min, was reverse transcribed in a final volume of 10 μl containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 μM random hexamers (Gibco/BRL) and 150 units M-MLV reverse transcriptase (Gibco/BRL). The reaction was allowed to proceed for 60 min at 37°C and then terminated by heating at 90°C for 5 min. One microliter of this reaction was amplified by PCR in a final volume of 10 μl containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs (dATP, dTTP, dGTP and [^{32}P]dCTP), 4 ng/μl of each primer, and 0.02 unit/μl of Taq DNA polymerase (Gibco/BRL). The primers used to amplify ER-β cDNA were: sense, 5′-TGCTTTGGTTTGGGTGATTGC-3′ (nucleotides 1164–1185); antisense, 5′-TTTGCTTTTACTGTCCTCTGC-3′ (nucleotides 1402–1423) [12]. The PCR conditions were 1 min 94°C, 30 s 58°C, and 30 s 72°C, for 30 cycles. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel using unlabeled dNTPs.

2.8. CAT assay

Since the action of estrogen agonists/antagonists bound to estrogen receptors (either ER-α or ER-β) activate downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected by a Lipofectamin method (Gibco/BRL) with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) (provided by Dr P. Chambon, IGBMC, France) or AP-1-tk-CAT (provided by Dr Ronald Evans, Howard Hughes Medical Institute, La Jolla, CA). Transient transfections were performed in the presence of 4 μg of a β-galactosidase expression vector to correct for differences in transfection efficiencies. After trans-

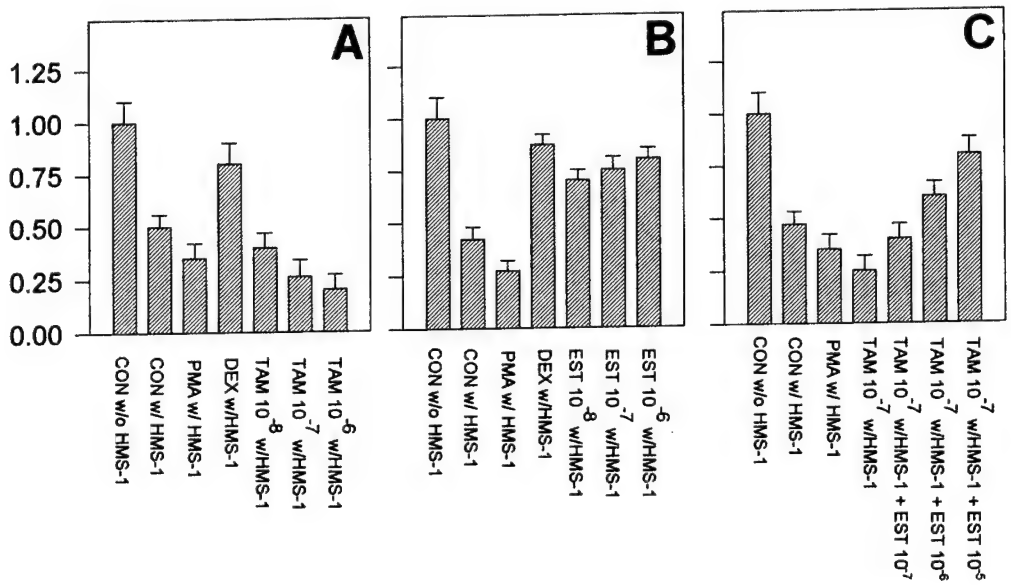

Fig. 1. Treatment of HMS-1 cell with estrogen antagonists/agonists at various concentrations of tamoxifen (A), 17β-estradiol (B), and various combinations (C) are compared with anti-invasive potentiating effects of PMA and anti-invasive abolishing effects of dexamethasone. The most highly invasive breast carcinoma cell line, MDA-MB-231, is depicted. Constitutive levels of invasion of these cells in the absence of intervening HMS-1 cells (CON without HMS-1) is assigned an arbitrary value of 1.0. The effects of HMS-1 cells (CON with HMS-1) and HMS-1 cells pretreated with various agents is expressed relative to this control level. The data represent mean ± SEM of three independent experiments. Results using other breast carcinoma cell lines and other myoepithelial cell lines were similar to results depicted for the MDA-MB-231 and HMS-1 cell lines, respectively.

fection, the cells were treated with 10^{-7} M tamoxifen, 10^{-7} M 17β-estradiol, or various concentrations and combinations, and harvested at the end of a 72-h incubation period. CAT activity was measured by thin layer chromatography by incubating the cell lysate at 37°C for 2 h with 4 μM [^{14}C]chloramphenicol and 1 mg/ml acetyl coenzyme A. Acetylated [^{14}C]chloramphenicol was quantitated.

2.9. Statistical analysis

Results were analyzed with standard tests of statistical significance, including the two-tailed Student's *t*-test and a one-way analysis of variance (ANOVA).

3. Results

3.1. Hormonal modulation of the anti-invasive phenotype of myoepithelial cells

HMS-1 and its CM (25×) exerted broad anti-invasive activity against both ER-positive (MCF-7 and T47D) and ER-negative (MDA-MB-231 and MDA-MB-468) lines. As reported previously, treatment of HMS-1 cells with different pharmacological agents, phorbol 12-myristate 13-acetate (PMA) (5 μM) and dexamethasone (0.25 μM) for 24 h had opposite effects on the anti-invasive phenotype. Dexamethasone nearly abolished the anti-invasive phenotype whereas PMA potentiated it. The effects of tamoxifen were similar to the anti-invasive potentiating effects of PMA (Fig. 1A). The anti-invasive potentiating effects of tamoxifen were dose-dependent (Fig. 1A). In contrast, 17β-estradiol reduced the anti-invasive effects of myoepithelial cells (Fig. 1B). Competition experiments between tamoxifen and 17β-estradiol showed that increasing concentrations of 17β-estradiol blocked the anti-invasive potentiating effects of tamoxifen (Fig. 1C).

3.2. Hormonal modulation of myoepithelial cell maspin and its anti-invasive effects

Immunoprecipitation of maspin from HMS-1 CM (Fig. 2A) reversed the anti-invasive effects of myoepithelial CM on breast carcinoma cell invasion in vitro (Fig. 2B). Tamoxifen treatment of HMS-1 resulted in a 2–3-fold increase in maspin secretion with increasing doses of tamoxifen (Fig. 3A) and increasing times of exposure (Fig. 3B). 17β-Estradiol, in contrast, exerted no effects on maspin secretion (Fig. 3C) and completely abolished the maspin stimulatory effects of tamoxifen in competition experiments (Fig. 3D). Tamoxifen's increase in maspin secretion was not due to an increase in steady state maspin mRNA levels which were essentially unchanged by this treatment (Fig. 3E).

3.3. Hormonal modulation of myoepithelial cell proliferation

Increasing and prolonged tamoxifen exposure resulted in striking anti-proliferative effects on myoepithelial cells (Fig. 4A) whereas increasing and prolonged 17β-estradiol exposure exerted no such

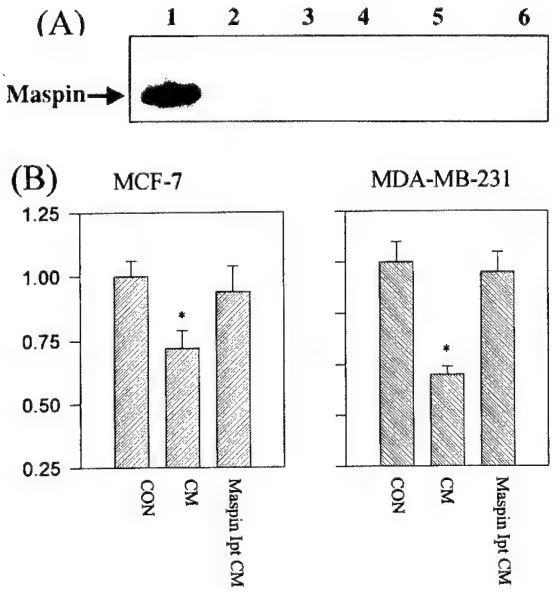

Fig. 2. (A) Maspin immunoprecipitation fraction at various dilutions of maspin antibody. Lane 1, 1/100; lane 2, 1/500; lane 3, 1/1000; lane 4, 1/2000. Optimal dilution was 1/100 to achieve nearly 100% immunoprecipitation. Other serpin antibodies used including anti-PAI-1 (lane 5) and anti-PAI-2 (lane 6) resulted in only negligible cross-reacting immunoprecipitation of maspin. (B) Effects of HMS-1 25 × CM and maspin-immunoprecipitated CM on breast carcinoma invasion. Control levels of invasion of designated breast carcinoma cell lines, MCF-7 and MDA-MB-231 were assigned arbitrary values of 1.0 and effects of CM and immunoprecipitated CM were expressed relative to these control levels. Results with other myoepithelial cell lines were similar. *Statistically significant differences compared with control ($P < 0.05$).

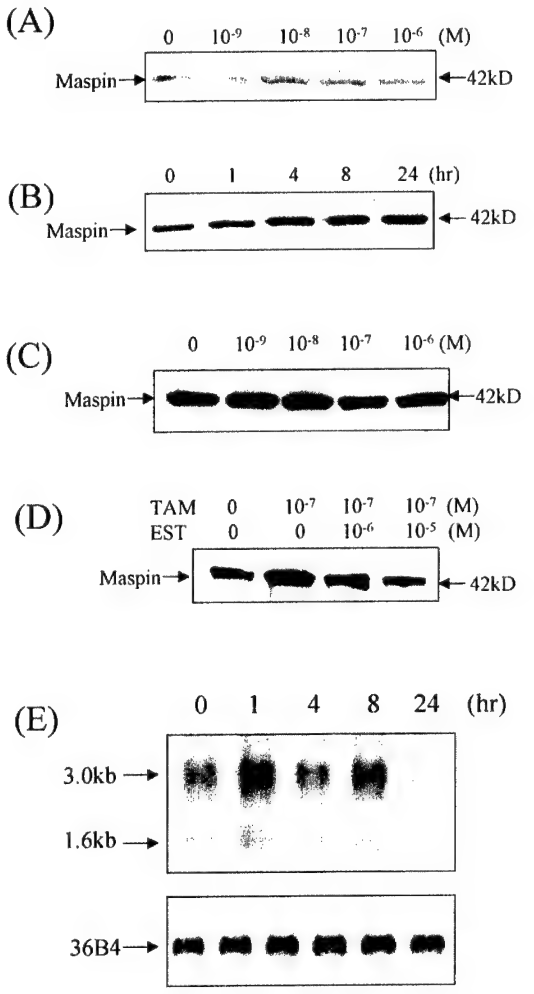

Fig. 3. (A) Dose response of tamoxifen (10^{-9}–10^{-6} M) treatment on maspin secretion over 24 h measured by Western blot with anti-maspin of HMS-1 CM (25×). (B) Effects of tamoxifen (10^{-7} M) treatment on maspin secretion for various times (h) of tamoxifen exposure. (C) Dose response of 17β-stradiol (10^{-9}–10^{-6} M) on maspin secretion by HMS-1 cells over 24 h. 17β-Estradiol exerted no stimulatory effects. (D) In competition experiments, increasing concentrations of 17β-estradiol completely blocked tamoxifen's stimulation of maspin secretion. (E) Northern blot of maspin steady-state mRNA levels following exposure of HMS-1 cells to tamoxifen (10^{-7} M) for indicated time periods. Two maspin transcripts (3.0, 1.6 kb) are present but no stimulatory effects of tamoxifen on maspin steady-state mRNA levels are seen. At 24 h, a slight decrease in steady-state mRNA levels was observed. Normalization was with 36B4. Results with other myoepithelial cell lines were similar.

effects (Fig. 4B) and blocked the anti-proliferative effects of tamoxifen in competition experiments (Fig. 4C). In the tamoxifen experiments only, a 3-fold increase in apoptotic index was observed; however, when the tamoxifen experiments were conducted in the presence of hydrocortisone (5 μM) to inhibit iNOS, no anti-proliferative effects and no increase in apoptotic index were observed (data not shown).

3.4. Hormonal modulation of myoepithelial cell NO and iNOS expression

Breast carcinoma CM (25×) was synergistic with tamoxifen but not 17β-estradiol in increasing myoepithelial cell NO production (Fig. 5A); this synergism was even more striking in co-culture experiments of MDA-MB-231 cells with HMS-1 cells (Fig. 5B). In both situations, 17β-estradiol blocked tamoxifen's synergistic effect on increased NO production (Fig. 5A,B). Tamoxifen together with MDA-MB-231 CM (25×) increased steady state iNOS mRNA levels over time (Fig. 5C,D). This effect was not observed with 17β-estradiol and was blocked by 17β-estradiol in competition experiments (data not shown). In the co-culture experiments with tamoxifen, an increase in apoptotic index was observed in both cell populations; however, this increase in apoptosis was blocked by the inhibition of iNOS and NO with the addition of hydrocortisone (5 μM) (data not shown).

3.5. Hormonal modulation of the myoepithelial cell's suppressor phenotype through ER-β

Myoepithelial cell lines lacked ER-α expression (Fig. 6A) but uniformly expressed ER-β (Fig. 6B). Since the action of estrogen agonists/antagonists bound to estrogen receptors (either ER-α or ER-β) activate downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) or AP-1-tk-CAT. Tamoxifen (10^{-7} M) increased AP-1-CAT activity 3-fold (Fig. 7A,B). This effect was not observed with 17β-estradiol. Furthermore 17β-estradiol (10^{-5} M) competed with and blocked the effects of tamoxifen (10^{-7} M) (Fig. 7A,B). 17β-Estradiol (10^{-7} M) did increase ERE-

CAT activity but tamoxifen (10^{-7} M) did not (Fig. 7C,D).

4. Discussion

Cancer cells come under the influence of important paracrine regulation from the host microenvironment [13]. Such host regulation may be as great a determinant of a tumor cell's behavior in vivo as the specific oncogenic or suppressor alterations occurring within the malignant cell itself and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves [14,15]. Both positive (fibroblast, myofibroblast and endothelial cell) and negative (tumor infiltrating lymphocytes, and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior in vivo [16,17]. One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group [1]. Our previous studies have shown that myoepithelial cells and derived cell lines exert suppressive effects on breast carcinoma cells through secretion of a number of different anti-invasive [1], anti-proliferative [3], and anti-angiogenic molecules [7]. Since issues of hormone replacement therapy and tamoxifen chemoprevention are timely, we wondered whether or not hormonal manipulations might affect myoepithelial cells directly in so far as their paracrine suppressive activities on breast cancer were concerned. In this study we specifically focused on two such myoepithelial-associated suppressor molecules: maspin and NO.

Treatment of myoepithelial cells with tamoxifen resulted in 2–3-fold increase of maspin, a recently identified tumor suppressor [18–21]. It had been shown in transfection studies that maspin was capable of inhibiting breast carcinoma cell growth, invasion, and metastasis in an autocrine manner [18–21]. Recently, it had also been demonstrated that rMaspin reduced the invasive phenotype of MDA-MB-435 cells by altering their integrin profile, particularly α5, which in turn converted these cells to a more benign epithelial phenotype, with less invasive ability [22]. We have recently demonstrated that maspin suppresses invasion in a paracrine manner [1]. Pharmacological enhancement of maspin secretion should therefore enhance tumor suppressor activity. In our study the anti-invasive effects of maspin were in evidence in both co-culture experiments and in CM experiments. In the co-culture experiments the invasive cells were placed in an upper chamber and an intervening layer of myoepithelial cells were placed in underlying Matrigel as previously reported [1]. Since myoepithelial cells were non-invasive, reversing the polarity of this system did not yield meaningful data. In the CM experiments 25 × CM was selected because in our previous studies [1], CM

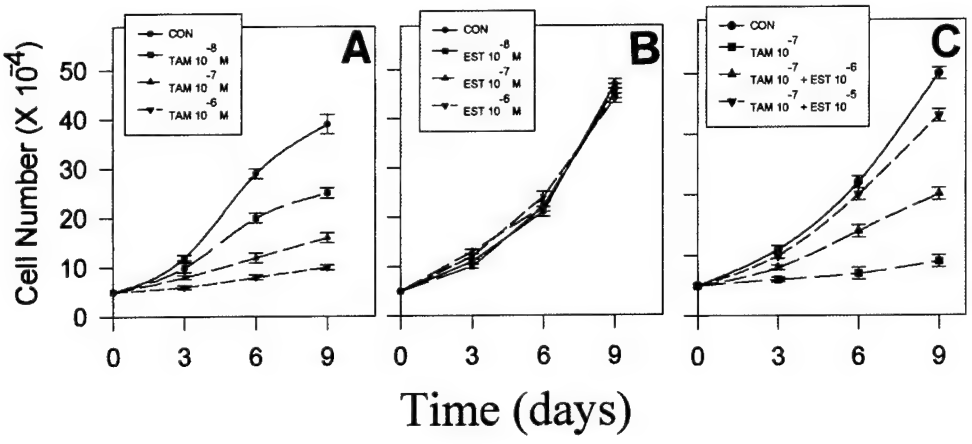

Fig. 4. Effects at various doses of tamoxifen (A), 17β-estradiol (B), and various concentrations and combinations (C) on HMS-1 cell proliferation. Tamoxifen inhibited proliferation, 17β-estradiol had no effect, and 17β-estradiol blocked tamoxifen's antiproliferative effects in competition experiments. The data represent mean ± SEM of three independent experiments. Results with other myoepithelial cell lines were similar.

had to be concentrated at least 25–100× for invasion-inhibitory activity to be observed. Our measurements of maspin in the co-culture system were approximately those of unconcentrated CM. The reason why the co-culture system with its intervening layer of myoepithelial cells was effective at inhibiting inva-

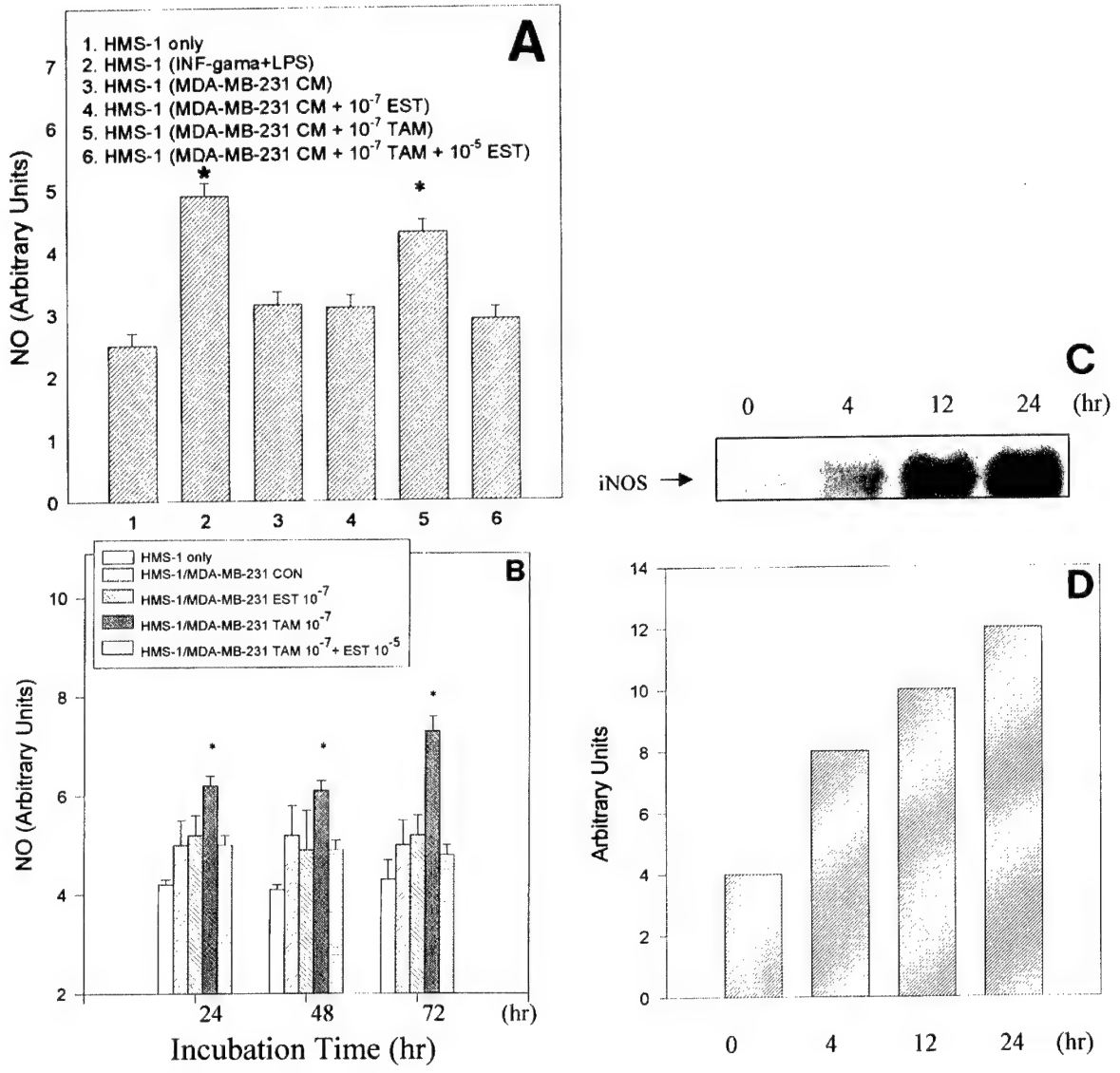

Fig. 5. (A) Nitric oxide (NO) activity in HMS-1 cells with various additions treated for 72 h. Lane 1, HMS-1 only; lane 2, HMS-1 incubated with the cytokine inducers INF-γ and LPS; lane 3, HMS-1 cells incubated with MDA-MB-231 CM (25×); lane 4, HMS-1 cells incubated with MDA-MB-231 CM (25×) plus 10^{-7} M 17β-estradiol; lane 5, HMS-1 cells incubated with MDA-MB-231 CM (25×) plus 10^{-7} M tamoxifen; lane 6, HMS-1 cells incubated with MDA-MB-231 CM (25×) plus 10^{-7} M tamoxifen and 10^{-5} M 17β-estradiol. *Statistically significant differences compared with control ($P < 0.05$). (B) NO activity in HMS-1 cells co-cultured with MDA-MB-231 cells treated with various additions for indicated times. Tamoxifen, but not 17β-estradiol, was effective at increasing NO production. 17β-Estradiol, furthermore, blocked tamoxifen's effect of increasing NO production. *Statistically significant differences compared with control. (C) iNOS expression by Northern blot in HMS-1 cells treated with MDA-MB-231 CM (25×) plus 10^{-7} M tamoxifen for increasing time periods (0–24 h). Results with other myoepithelial cell lines were similar. (D) Densitometric analysis of bands of iNOS expression by Northern blot.

sion is probably because the myoepithelial cells provide a gradient of maspin that the invading tumor cells encounter as they approach. So, in the co-culture system, the concentration of maspin in the invading tumor cell's microenvironment, may, in fact, be much greater than in the 25 × CM experiments.

NO is derived from L-arginine by constitutive nitric oxide synthase (NOS) or inducible nitric oxide synthase (iNOS). NO produces multiple suppressive effects on cancer cells. Recently, it has been shown that the production of endogenous NO is associated with apoptosis of tumor cells, suppression of tumorigenicity, and the abrogation of metastasis [23,24]. In the present study, we have demonstrated that iNOS is present in myoepithelial cells and both iNOS expression and NO production is increased by tamoxifen in the presence of breast carcinoma cell CM. Tamoxifen also exerts antiproliferative effects on myoepithelial cells. These findings implicate NO as another important suppressor molecule made by myoepithelial cells that is hormonally regulated. To confirm these observations we have provided a functional assay to demonstrate NO action. We have conducted additional experiments using hydrocortisone (5 μM), an inhibitor of iNOS. We have demonstrated that in this setting the anti-proliferative effects of tamoxifen on myoepithelial cells are not observed. In addition in the co-culture experiments where the MDA-MB-231 line was mixed with HMS-1 cells, in the presence of tamoxifen we noted increased apoptosis after 72 h in both populations of cells. However, in the presence of hydrocortisone (5 μM), this increased apoptosis was not observed. In both the HMS-1 growth experiments and in the co-culture experiments, the inhibition of iNOS by hydrocortisone did not affect maspin levels. Although these studies are preliminary, they suggest that the increased NO released by myoepithelial cells in response to tamoxifen has functional significance in terms of anti-proliferative and apoptotic tumor suppressive effects.

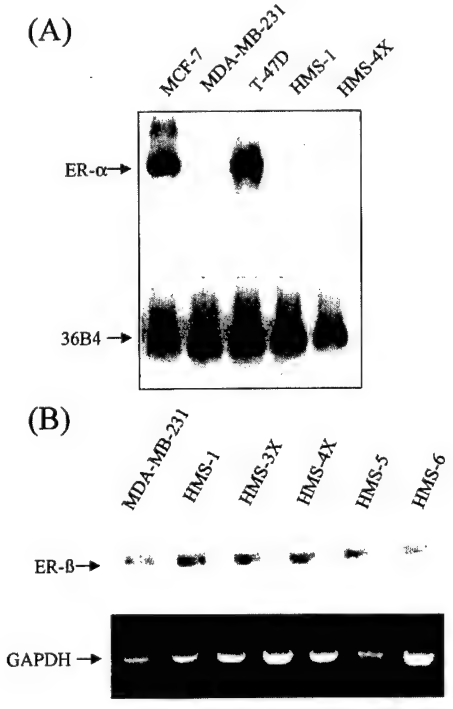

Fig. 6. Expression of ER-α and ER-β in representative myoepithelial cell lines. (A) Northern blot analysis, ER-α expression. Normalization was with 36B4. (B) ER-β expression by RT–PCR. GAPDH served as a housekeeping control.

Estrogen agonists and antagonists can exert effects on their cellular targets through a number of mechanisms: some of these are rapid direct membrane effects of the hormone on ion channels [25] or on the induction of susceptibility to oxidative stresses that do not require receptor binding and downstream transcriptional activation of genes. Other effects of estrogen agonist and antagonist action are mediated through receptor binding, either ER-α or ER-β. Our myoepithelial cells completely lacked expression of ER-α but expressed ER-β. Estrogen action was initially thought to mediated principally through a single ER, ER-α, a member of the steroid/thyroid/retinoic acid receptor superfamily [26]. Recently, however, a second ER, ER-β was identified in the rat, mouse, and human [27,28]. ER-β shares a similar structural and functional composition with ER-α and has been shown to activate the transcription of similar ERE-containing target genes [27,28]. However 17β-estradiol and tamoxifen have been shown to differentially activate other ER downstream genes containing ER-dependent AP-1 response elements [29]. In our study it was important to distinguish which mode of action was likely responsible for tamoxifen's enhancement of the anti-invasive phenotype of myoepithelial cells and the enhanced secretion of maspin and production of NO. The fact that 17β-estradiol competed with tamoxifen in blocking all of its actions on myoepithe-

lial cells including its anti-invasive potentiating effects, its stimulation of effector molecules: maspin and NO, and its transcription-activation of an ER-dependent AP-1 response element supports that tamoxifen's mode of action on myoepithelial cells is through the estrogen receptor, in this case, ER-β. In our study tamoxifen, but not 17β-estradiol upregulated AP-1-CAT activity but not ERE-CAT activity. These findings suggest that the downstream events of iNOS expression, NO production, and maspin secretion are mediated through tamoxifen's binding to ER-β and subsequent ER-β–AP-1, rather than ER-β–ERE, *trans*-activation.

The effects of HRT and tamoxifen chemoprevention are timely issues for patients at risk for developing breast cancer. It must be emphasized that all of these exogenous hormones exert multiple effects on multiple organ systems, some desired, some untoward that have global implications [30,31]. It is likely that not all of the effects of these hormones have been defined. The present study has examined the effects of tamoxifen and 17β-estradiol on myoepithelial cells in vitro. It is interesting that whereas tamoxifen increased the anti-invasive and tumor-suppressive effects of myoepithelial cells by maspin and NO-dependent mechanisms, 17β-estradiol reduced the anti-invasive effects of myoepithelial cells by maspin and NO-independent mechanisms. We can only speculate at this point concerning these latter mechanisms. In our previous study [1], we noted that dexamethasone similarly reduced the anti-invasive effects of myoepithelial cells by a maspin-independent mechan-

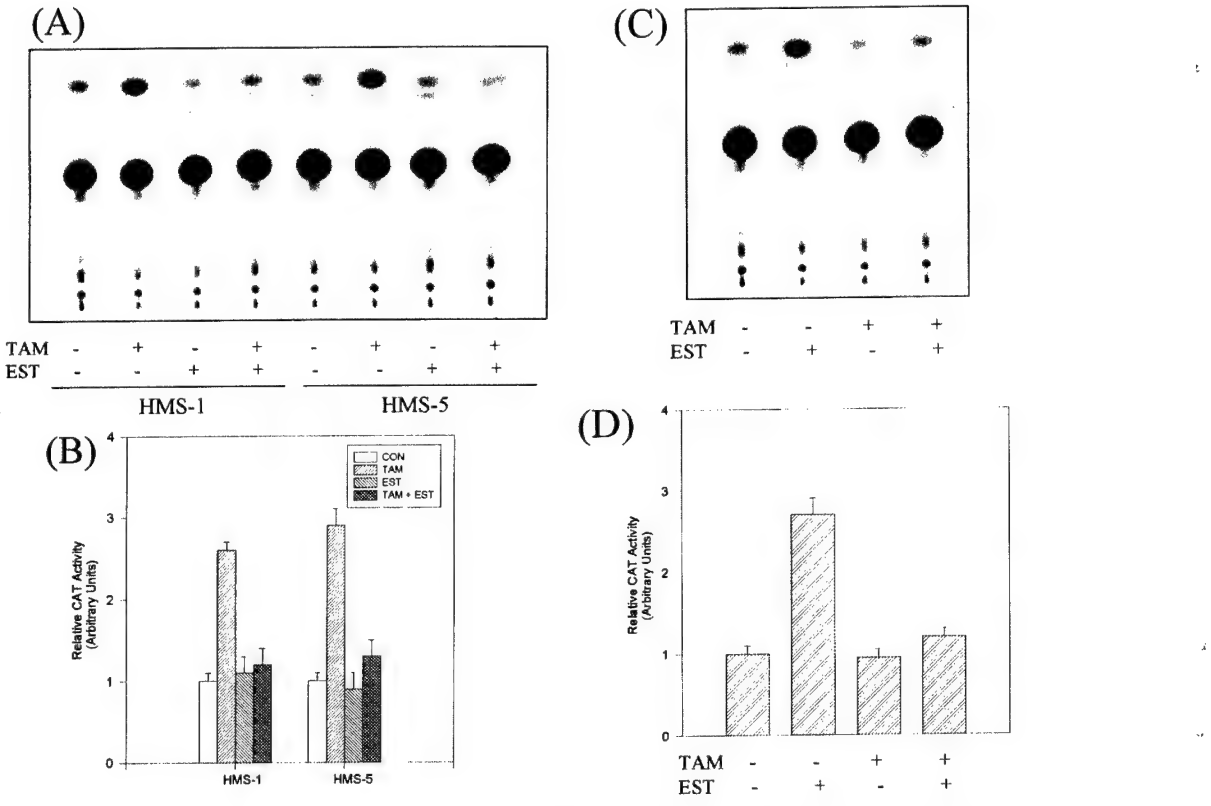

Fig. 7. (A) Tamoxifen (10^{-7} M) stimulation of AP-1-CAT activity in representative myoepithelial cell lines, HMS-1 and HMS-5. 17β-Estradiol (10^{-7} M) exerted no such stimulatory effects and blocked the effects of tamoxifen at high doses (10^{-5} M). (B) Results depicted are the means of three independent experiments. Error bars represent standard errors. (C) Tamoxifen (10^{-7} M) exerted no stimulation of ERE-CAT activity in HMS-1 whereas 17β-estradiol (10^{-7} M) stimulated a 2–3-fold increase in ERE-CAT which was blocked by tamoxifen (10^{-5} M). (D) Results depicted are the means of three independent experiments. Error bars represent standard errors.

ism. So possibly 17β-estradiol and dexamethasone act similarly. It should be pointed out that in our hormonal studies with 17β-estradiol, although we used amounts comparable to the amounts used in numerous other in vitro studies, the amounts used (10^{-8}–10^{-6} M) reflect non-physiological doses. Their relevance to HRT issues can therefore be questioned as the level of active hormones in HRT would not be expected to get beyond an equivalence of 10^{-9} M. The tamoxifen amounts (10^{-8}–10^{-6} M) we used in our in vitro experiments, in contrast, were not above what is found in patients [32] and our observations with tamoxifen may more likely have clinical relevance.

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The human myoepithelial cell displays a multifaceted anti-angiogenic phenotype

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Human myoepithelial cells which surround ducts and acini of certain organs such as the breast form a natural border separating epithelial cells from stromal angiogenesis. Myoepithelial cell lines (HMS-1-6), derived from diverse benign myoepithelial tumors, all constitutively express high levels of active angiogenic inhibitors which include TIMP-1, thrombospondin-1 and soluble bFGF receptors but very low levels of angiogenic factors. These myoepithelial cell lines inhibit endothelial cell chemotaxis and proliferation. These myoepithelial cell lines sense hypoxia, respond to low O_2 tension by increased HIF-1α but with only a minimal increase in VEGF and iNOS steady state mRNA levels. Their corresponding xenografts (HMS-X-6X) grow very slowly compared to their non-myoepithelial carcinomatous counterparts and accumulate an abundant extracellular matrix devoid of angiogenesis but containing bound angiogenic inhibitors. These myoepithelial xenografts exhibit only minimal hypoxia but extensive necrosis in comparison to their non-myoepithelial xenograft counterparts. These former xenografts inhibit local and systemic tumor-induced angiogenesis and metastasis presumably from their matrix-bound and released circulating angiogenic inhibitors. These observations collectively support the hypothesis that the human myoepithelial cell (even when transformed) is a natural suppressor of angiogenesis. *Oncogene* (2000) **19,** 3449–3459.

Keywords: thrombospondin-1; hypoxia inducible factor-1; angiogenic inhibitors; angiogenic factors

Introduction

Cancer cells come under the influence of important paracrine regulation from the host microenvironment (Cavenee, 1993). Such host regulation may be as great a determinant of tumor cell behavior *in vivo* as the specific oncogenic or tumor suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, growth factors, or host cells (Liotta *et al.*, 1991; Safarians *et al.*, 1996). Both positive (fibroblast, and endothelial cell) and negative (tumor infiltrating lymphocyte and macrophage) cellular regulators exist which profoundly affect tumor cell behavior *in vivo* (Folkman and Klagsbrun, 1987; Cornil *et al.*, 1991). One host cell, the myoepithelial cell, has escaped attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, contributes largely to the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and differentiating epithelial cells in health and to abnormally proliferating and differentiating epithelial cells in precancerous disease states such as ductal carcinoma *in situ* (DCIS) of the breast. This anatomical relationship suggests that myoepithelial cells may exert important paracrine effects on normal glandular epithelium and may negatively regulate the progression of DCIS to invasive breast cancer. Previous studies of our laboratory have demonstrated that the human myoepithelial cell exerts multiple tumor suppressive effects on breast carcinoma cells inhibiting both cellular invasion and proliferation as well as inducing apoptosis (Sternlicht *et al.*, 1996a,b, 1999; Shao *et al.*, 1998). The inhibition of invasion is mediated predominantly by myoepithelial maspin (Sternlicht *et al.*, 1997; Shao *et al.*, 1998). In these previous studies (Sternlicht *et al.*, 1996a,b, 1997; Shao *et al.*, 1998), we used our established immortalized human myoepithelial cell lines and transplantable xenografts derived from benign or low grade human myoepitheliomas of the salivary gland, bronchus and breast. These cell lines and xenografts, though transformed, express nearly identical myoepithelial markers and gene products as their normal *in situ* counterparts and display an essentially normal diploid karyotype (Sternlicht *et al.*, 1996b). Furthermore they have maintained strong myoepithelial marker expression of S100 protein, smooth muscle actin, and cytokeratins over 100 passages. Unlike the vast majority of human tumor cell lines and xenografts which exhibit matrix-degrading properties these myoepithelial lines/xenografts like their normal myoepithelial counterparts *in situ* retain the ability to secrete and accumulate an abundant extracellular matrix composed of both basement membrane and non-basement membrane components. When grown as monolayers these myoepithelial cell lines exert profound and specific effects on normal epithelial and primary carcinoma morphogenesis (Sternlicht *et al.*, 1996b). These studies support that our established myoepithelial lines/xenografts recapitulate a normal differentiated myoepithelial phenotype and can therefore be used experimentally as a primary myoepithelial surrogate.

Because one other important cornerstone of tumor suppression would be a suppressive effect on angio-

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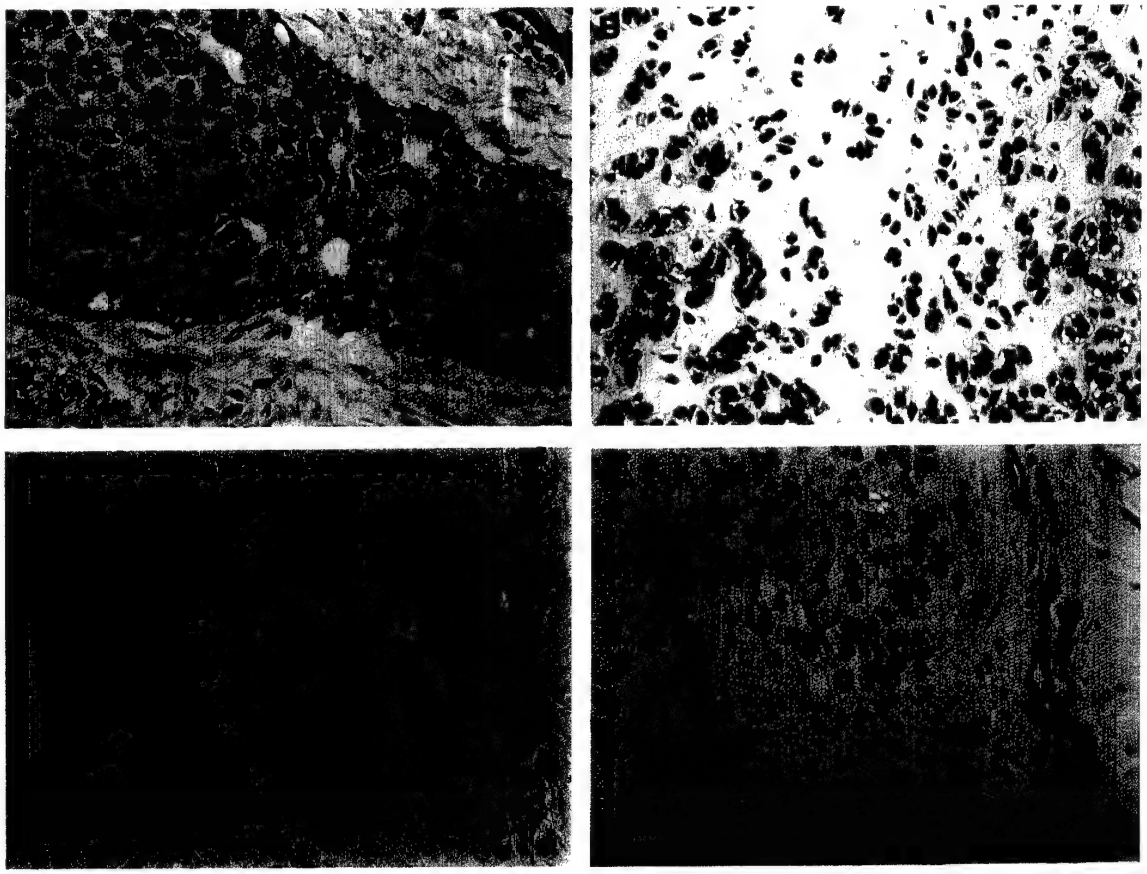
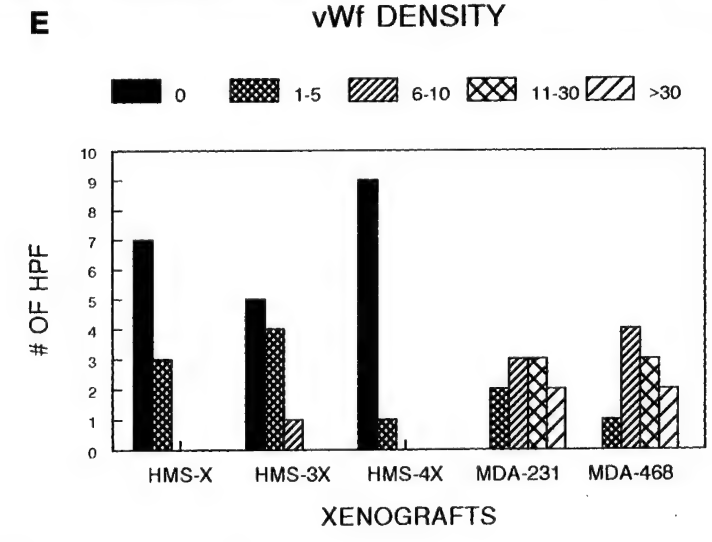
genesis and because the main product of the myoepithelial cell, the basement membrane, is thought to be a natural reservoir of angiogenic inhibitors (Folkman, 1990, 1995b; Weidner *et al*., 1991; Fridman *et al*., 1991), we decided in the present study to investigate whether myoepithelial cells naturally display an anti-angiogenic phenotype. Because angiogenesis is now such an important biological phenomenon having implications in wound healing, inflammation and tumor biology, discovering and characterizing a new natural form of negative regulation of angiogenesis would have implications in all of these diverse areas of investigation.

Results

Myoepithelial cells *in situ* separate epithelial cells from stromal angiogenesis and this seemingly banal observation serves to illustrate the fact that stromal angiogenesis never penetrates this myoepithelial barrier (Figure 1a) raising the hypothesis that myoepithelial cells are





natural suppressors of angiogenesis. This observation was re-inforced by a microscopic, immunohistochemical and DNA analysis of our myoepithelial xenografts. Our diverse myoepithelial xenografts secrete and accumulate an abundant extracellular matrix which is devoid of blood vessels in routine hematoxylin and eosin staining (Figure 1b) and vWf immunocytochemical staining (Figure 1c) in contrast to non-myoepithelial xenografts which show bursts of blood vessels (Figure 1d). Quantitation of vessel density in 10 H.P.F.'s reveals absent to low vessel density in the myoepithelial xenografts compared to the non-myoepithelial xenografts ($P<0.01$) (Figure 1e). Murine DNA Cot-1 analysis further reveals the absence of a murine component in the myoepithelial xenografts. Since in the xenografts, angiogenesis would be murine in origin, the absence of a murine DNA component is another indication that angiogenesis is minimal. Interestingly the myoepithelial xenografts grew slowly compared to the non-myoepithelial xenografts (Figure 1f), a feature which was not found in comparisons between the myoepithelial *versus* non-myoepithelial cell lines (data not shown).

To explain these *in vivo* observations, we analysed the gene expression profiles of our myoepithelial cell lines *versus* non-myoepithelial cell lines with respect to known angiogenic inhibitors and angiogenic factors. HMS-1, as a prototype myoepithelial cell line, constitutively expressed none or very low levels of the known angiogenic factors including bFGF, aFGF, angiogenin, TFGα, TGFβ, TNF-α, VEGF, PD-ECGF, PlGF, IFα, HGF, and HB-EGF but rather expressed thrombospondin-1, TIMP-1 and soluble bFGF receptors at high levels; this was in contrast to a high angiogenic factor (which included bFGF, VEGF, TFGα, TGFβ, HB-EGF, and PD-ECGF) to angiogenic inhibitor gene expression profile which was observed in non-myoepithelial cell lines (Figure 2a). Other

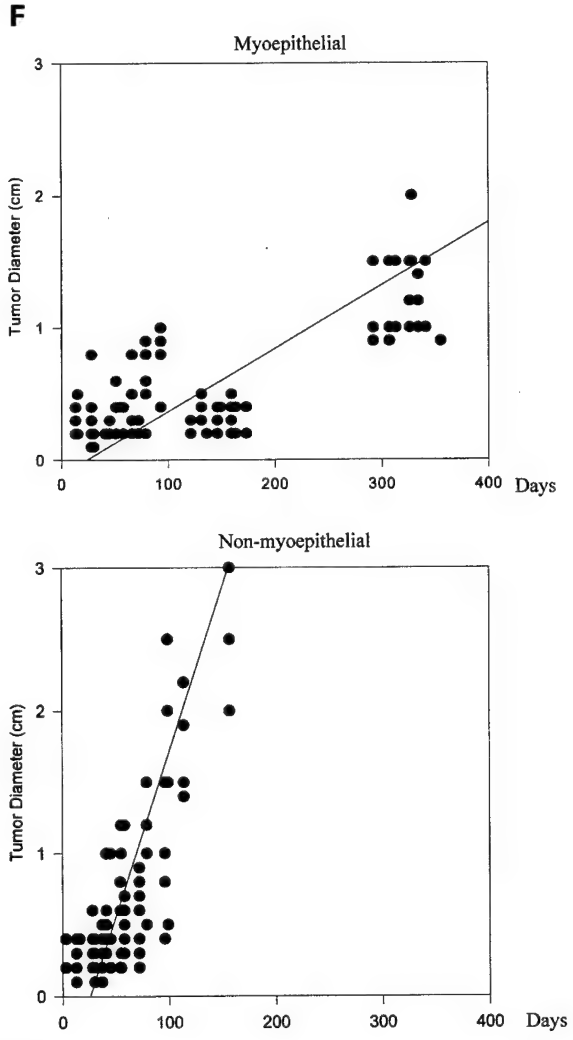


Figure 1 (**a**) A layer of myoepithelial cells immunoreactive for maspin demarcates the separation of proliferating epithelial cells from underlying stroma in this case of DCIS. Angiogenesis which occurs in the stroma never penetrates this layer of myoepithelial cells. Human myoepithelial xenografts, for example, HMS-X (**b**) exhibit an abundant extracellular matrix devoid of blood vessels. Absent vWf immunoreactivity is depicted in HMS-4X (**c**) compared to strong vWf immunoreactivity in the non-myoepithelial xenograft, MDA-MB-231 (**d**). Density of vWf positive vessels in 10 H.P.F.'s of myoepithelial *versus* non-myoepithelial xenografts reveals absent to significantly fewer blood vessels in the former xenografts (**e**). Growth rates of myoepithelial xenografts, for example, HMS-X, compared to growth rates of non-myoepithelial xenografts, for example, MDA-MB-231, revealed comparatively slow myoepithelial growth (**f**), suggesting a link to endogenously low levels of angiogenesis

myoepithelial cell lines (HMS-2-6) exhibited an angiogenic inhibitor/angiogenic factor profile similar to that of HMS-1. Interestingly in 2 M urea extracts of the myoepithelial xenografts but not in any of the non-myoepithelial xenografts, strong thrombospondin-1, TIMP-1 as well as plasminogen and prolactin fragments could be detected by Western blot (Figure 2a). HMS-1 and HMS-1 CM (concentrated 10–100-fold) exerted a marked inhibition of endothelial migration (Figure 2b) and proliferation (Figure 2c), both of which were abolished by pretreatment of the myoepithelial cells with cyclohexamide or dexamethasone. HMS-1 cells themselves did not migrate in response to either K-SFM, FCS, or bFGF. When mixed with UVE, HMS-1 cells reduced endothelial migration to $12 \pm 6\%$ of control ($P<0.01$). HMS-1 concentrated CM reduced migration to $8 \pm 7\%$ of control ($P<0.01$). All of the non-myoepithelial malignant human cell lines studied stimulated both endothelial migration and proliferation. Concentrated CM from HMS-1, when fractionated on a heparin-Sepharose column, inhibited endothelial proliferation to $47 \pm 10\%$ of control ($P<0.01$). This inhibitory activity was present only in the 1.5–2.0 M gradient fraction (Figure 2d). Pretreatment of HMS-1 cells with PMA resulted in a 2–5-fold increase in endothelial antiproliferative inhibitory activity in both unfractionated CM (Figure 2c) as well as in the heparin-Sepharose fraction. Western blot of

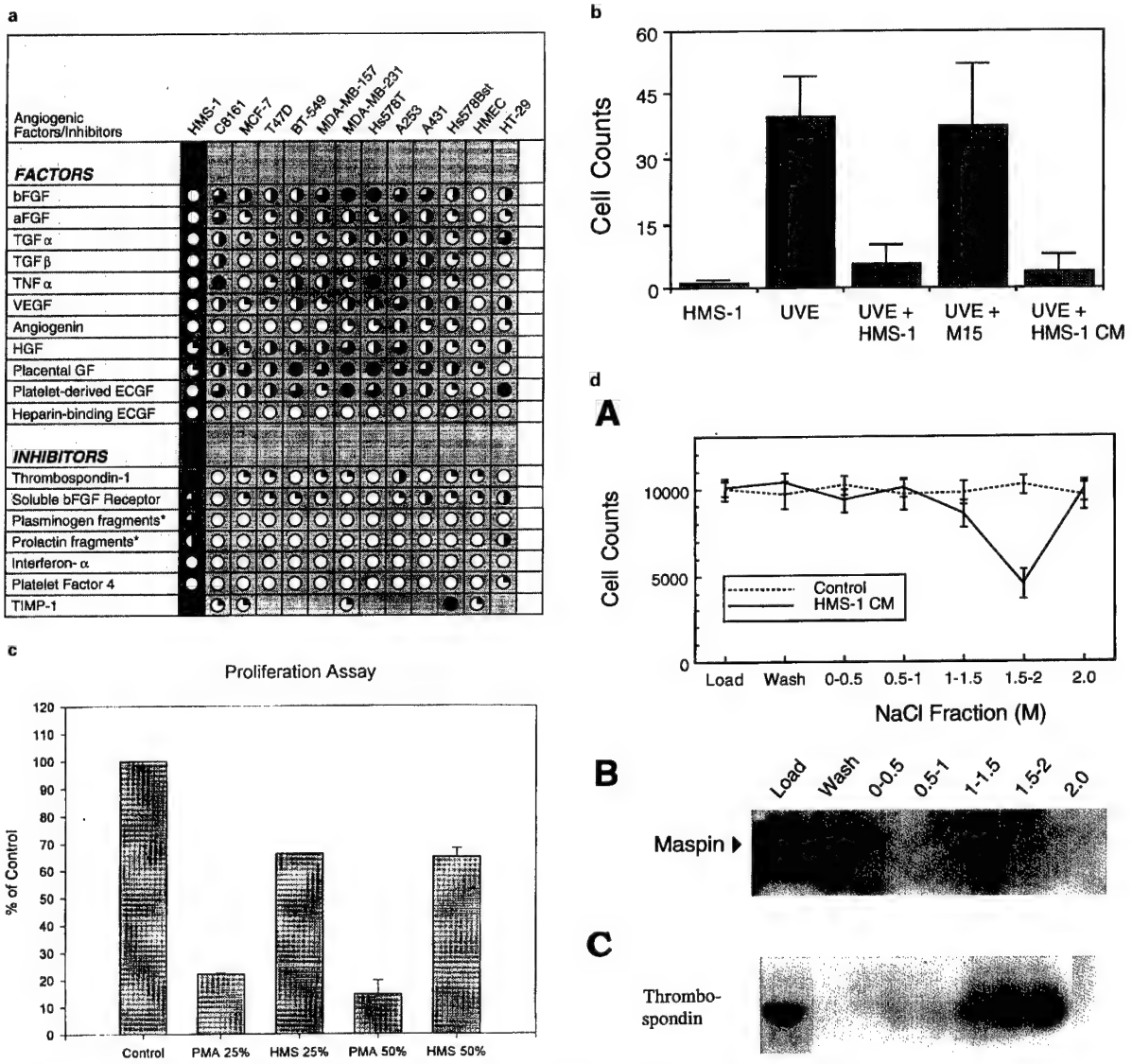


Figure 2 (**a**) Relative constitutive gene expression profiles of diverse angiogenic inhibitors and angiogenic factors in HMS-1 compared to numerous other non-myoepithelial cell lines. All measurements were made by Western blot on either CM or matrix extracts* and depicted as relative levels of expression. HMS-1 (HMS-X*) uniquely expressed a balance of angiogenic inhibitors over angiogenic factors. (**b**) Inhibition of UVE chemotaxis by HMS-1 cells and HMS-1 CM is depicted as cell counts collected on the undersurface of a dividing filter. HMS-1 cells themselves were non-migratory. A control non-myoepithelial human melanoma cell line, M15, did not inhibit UVE chemotaxis. (**c**) HMS-1 CM (percentage of CM added) inhibition of UVE proliferation is depicted. PMA pretreatment of HMS-1 increased antiproliferative activity. (**d**) Fractionation of the HMS-1 CM on a heparin-Sepharose column revealed peak antiproliferative activity in the 1.5–2.0 NaCl fraction [A], a fraction devoid of maspin [B] but containing thrombospondin-1 [C]

the heparin-Sepharose column fractions revealed the presence of maspin in the load and wash fractions only and not in the 1.5–2.0 M fraction; in contrast the 1.5–2.0 M NaCl fraction contained thrombospondin-1 (Figure 2d). Immunoprecipitation of this fraction with anti-thrombospondin was effective at removing all thrombospondin-1 but decreased endothelial antiproliferative activity by only 50% raising the possibility that other angiogenic inhibitors as yet uncharacterized were present in this fraction. The other myoepithelial cell lines (HMS-2-6) exhibited similar anti-angiogenic inhibitory activity in their fractionated and unfractionated CM.

To further explain our *in vivo* observations of minimal angiogenesis in our myoepithelial xenografts, *in vitro* and *in vivo* hypoxia studies were carried out. Non-myoepithelial xenografts, e.g. MDA-MB-231 exhibited florid hypoxia but only minimal necrosis (Figure 3a) when they reached a size of 2.0 cm. In contrast, the myoepithelial xenografts exhibit only minimal hypoxia but prominent necrosis ($P<0.001$) (Figure 3b,c) at the same size of 2.0 cm. Quantitation of the areas of hypoxia (pimonidazole immunoreactivity) and areas of necrosis (Figure 3d) in the myoepithelial *versus* non-myoepithelial xenografts suggest that in the myoepithelial tumors where angiogenesis is minimal hypoxic areas progress to necrosis rapidly whereas in the non-myoepithelial tumors hypoxic areas accumulate but do not progress to necrosis presumably from the angiogenesis which the hypoxia elicits. Comparative analysis of myoepithelial *versus* non-myoepithelial cell lines to low O_2 tension reveals that while both cell lines sense hypoxia in that they respond by increasing HIF-1α (Figure 3e), the myoepithelial lines upregulate their steady state mRNA levels of the downstream genes, VEGF (Figure 3f) and iNOS (Figure 3g) to a lesser extent than the carcinoma lines suggesting the possibility of decreased transactivation of HRE. Specifically we observed an approximate 1.7-fold increase in VEGF (1.1-fold increase in iNOS) in myoepithelial cells in response to hypoxia compared to an approximate 2.5-fold increase in VEGF (1.5-fold increase in iNOS) in carcinoma cell lines in response to hypoxia. Although these fold differences by themselves are not impressive, the absolute levels of VEGF (and iNOS) expressed in carcinoma cells in response to hypoxia are 2.5-fold greater for VEGF (and 1.7-fold greater for iNOS) than the levels of VEGF (and iNOS) expressed in myoepithelial cells in response to hypoxia. Therefore it can be concluded that myoepithelial cells do not express VEGF or iNOS in response to hypoxia to nearly the same extent as carcinoma cells.

To study both local and systemic effects of myoepithelial cells on metastasis, spontaneously metastasizing tumor cells were injected into our myoepithelial xenografts. The highly metastatic *neo*C8161 cells injected into the myoepithelial xenografts could be recovered in significant numbers although the numbers of clones recovered were less than those recovered from the nonmyoepithelial xenografts. Histological analysis of the extirpated xenografts revealed *neo*C8161 cells actively invading through all of the nonmyoepithelial xenografts in contrast to the appearance within the myoepithelial xenografts where the *neo*C8161 cells were confined to the immediate areas around the injection site. Pulmonary metastases of *neo*C8161 were completely absent in the myoepithelial xenograft-injected group whereas they were quite numerous in the nonmyoepithelial group ($P<0.001$). Analysis of extirpated myoepithelial xenografts containing injected *neo*C8161 cells contained no evidence of murine angiogenesis by either vWf immunocytochemical studies or murine DNA Cot-1 analysis whereas a similar analysis of extirpated *neo*C8161 injected-nonmyoepithelial xenografts showed an increase in murine angiogenesis by both methods (data not shown). This suggested that either the matrices of our myoepithelial xenografts or gene product(s) of the myoepithelial cells or both were inhibiting *neo*C8161-induced angiogenesis *in vivo*. We, in fact, found evidence of thrombospondin-1, TIMP-1, soluble bFGF receptors, prolactin and plasminogen fragments within 2 M urea extracts of our myoepithelial xenografts (Figure 4a). In tail vein injection studies of *neo*C8161, in mice harboring the myoepithelial xenografts, *neo*C8161 formed smaller pulmonary colonies than in mice harboring nonmyoepithelial xenografts or in control mice (no xenografts) ($P<0.01$) (Figure 4b,c,d). In a vWf factor immunocytochemical analysis of these smaller colonies in the mice harboring the myoepithelial xenografts, angiogenesis was minimal (data not shown).

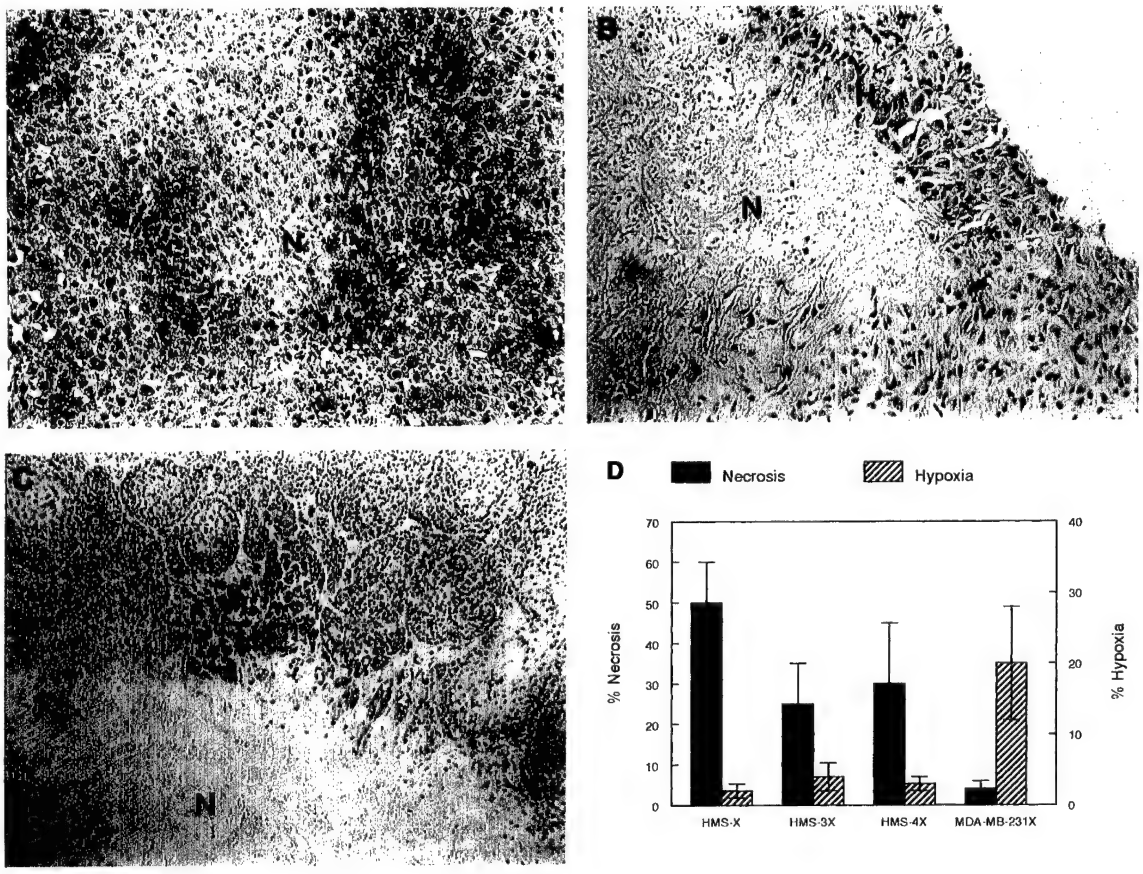
Discussion

Blood vessel proliferation (angiogenesis) occurs in a number of disease states including inflammation and neoplasia and both positive and negative regulators of angiogenesis exist that influence this process. However angiogenesis is not ubiquitous and certain tissues such as mature cartilage are resistant to this process. Angiogenesis also does not occur on the epithelial side of the basement membrane even in proliferative and precancerous disease states which stimulate angiogeneis in the underlying stroma. The border between epithelial cells and stroma is lined by myoepithelial cells suggesting that these cells may exert negative regulation on the angiogenic process. Our studies, in fact, provide evidence that human myoepithelial cells are natural suppressors of angiogenesis and display this phenotype in a multifaceted manner.

Tumor angiogenesis, like tumor invasion, is thought to be determined by the balance of natural positive and negative regulators which occur within the tumor's microenvironment (Tobacman, 1997; Xiao *et al*., 1999; Folkman, 1995a; Hanahan and Folkman, 1996). Although certain specific angiogenic inhibitors can be expressed by malignant cell lines, the vast majority of malignant cell lines secrete both more angiogenic factors than inhibitors and a greater molar ratio of angiogenic factors to inhibitors. Situations, both natural and therapeutic, which shift the overall balance to an excess of angiogenic inhibitors over angiogenic factors would be situations which are tumor suppressive. All of our myoepithelial cell lines constitutively expressed a high ratio of angiogenic inhibitors to angiogenic factors. The net effects of myoepithelial cells and their CM inhibited endothelial migration; in contrast the net effects of the non-myoepithelial malignant human cell lines studied all stimulated endothelial migration and proliferation. Heparin-Sepharose fractionated CM from our myoepithelial cell

lines demonstrated a marked inhibition of endothelial proliferation in the 1.5–2.0 M NaCl gradient fraction, a fraction containing thrombospondin-1 instead of maspin. Thrombospondin immunoprecipitation experiments performed on this fraction suggested that the mechanism of antiangiogenesis, in part, involved thrombospondin-1. It should be pointed out that our assays for antiangiogenic activity utilized human umbilical vein endothelial cells (commonly called HUVECs), cells which lack one of the receptors, CD36, to which thrombospondin-1 binds (Dawson *et al.*, 1997). However another study found proliferation in HUVECs to be inhibited by thrombospondin-1 (Bagavandoss and Wilkes, 1990). The implication of these studies to our present work is that our use of HUVECs might have resulted in an underestimate of the thrombospondin-1 mediated antiangiogenic activity of CM from myoepithelial cell lines. Our use of microvascular endothelial cells might be more appropriate for our future studies. It is also interesting that in our human umbilical vein endothelial proliferation assays, maspin did not appear to have antiangiogenic activity. Maspin has recently been observed, in fact, to be an angiogenesis inhibitor (Zhang *et al.*, 2000). In that study, the antiangiogenic effects of maspin were observed against human microvascular endothelial cells and not human umbilical vein endothelial cells. Our previous studies have shown that myoepithelial cells secrete high levels of maspin both *in vitro* and *in vivo* (Sternlicht *et al.*, 1997). Myoepithelial maspin can hence be added to the growing list of angiogenic inhibitors produced selectively and at high levels by myoepithelial cells. Interestingly both maspin and thrombospondin-1 have been shown to be autocrine tumor suppressors in experiments involving their respective cDNAs transfected into breast carcinoma cell lines (Zou *et al.*, 1994; Weinstat-Saslow *et al.*, 1994). In our past and present studies we argue for a role of both molecules as paracrine tumor suppressors elaborated by myoepithelial cells. The similarly enhancing effect of PMA on both invasion inhibition demonstrated in a previous study (Sternlicht *et al.*, 1997) and angiogenesis inhibition demonstrated in the present study argues that PMA pleiotropically promotes the natural suppressor effects of myoepithelial cells.

There was minimal angiogenesis in our myoepithelial xenografts which also exhibited a very slow growth rate compared to their non-myoepithelial counterparts. This growth rate difference was not observed between the myoepithelial *versus* non-myoepithelial cell lines. It is attractive then to postulate that the slow growth of the myoepithelial xenografts is causally related to their minimal angiogenesis. To explain this minimal myoepithelial angiogenesis, we reasoned that there were three possible mechanisms. The first was that myoepithelial cells expressed a balance of angiogenic inhibitors over angiogenic factors so that the myoepithelial tumor's microenvironment was inhibitory to angiogenesis. The second possible mechanism was that myoepithelial cells, like chondrocytes, either do not sense hypoxia or are resistant to the effects of hypoxia. The third possible mechanism was that myoepithelial cells



experience hypoxia but are not able to respond to it by inducing angiogenesis. Our studies revealed that both the first and third mechanisms are operating within myoepithelial cells but that the second mechanism is not: myoepithelial cells secrete a balance of angiogenic inhibitors over angiogenic factors, they do however sense hypoxia but are not able to respond effectively downstream to hypoxia.

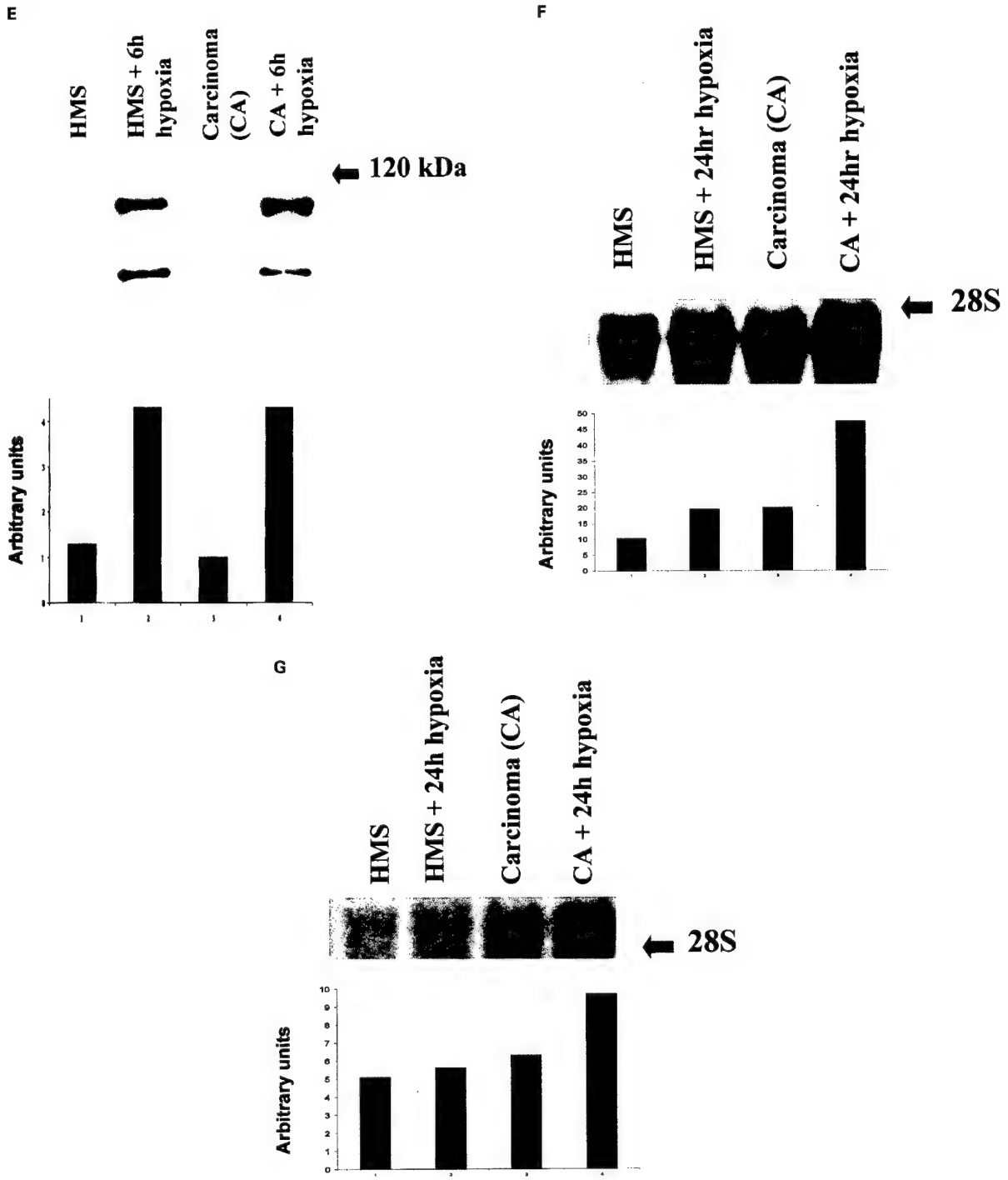


Figure 3 Pimonidazole immunoreactivity as a measure of hypoxia is prominently contrasted in a non-myoepithelial xenograft, the MDA-MB-231 (**a**), *versus* two myoepithelial xenografts, HMS-4X (**b**) and HMS-X (**c**). Only a thin rim of pimonidazole immunoreactivity (H) is present in the myoepithelial xenografts but instead large adjacent areas of frank necrosis (N) are conspicuous (**b**)(**c**). Necrosis (N) in the MDA-MB-231, in contrast, is inconspicuous but hypoxia (H) is prominent (**a**). Per cent hypoxia and per cent necrosis in the myoepithelial *versus* non-myoepithelial xenografts are contrasted (**d**). In the myoepithelial xenografts necrosis is prominent whereas hypoxia is inconspicuous where the reverse is true in the non-myoepithelial xenografts. Under low O_2 tension, myoepithelial cells, e.g. HMS-1 (HMS), like non-myoepithelial carcinoma cells, e.g., MDA-MB-231 (CA), show an increase in HIF-1α (**e**) but, unlike carcinoma cells (CA), show less of an increase in VEGF (**f**) and iNOS (**g**) steady state mRNA levels. Other myoepithelial and carcinoma lines tested exhibited a similar pattern of findings

The specific gene products of myoepithelial cells per se may not be the sole determinants of this cell's antiangiogenic phenotype because these gene products may undergo extracellular modifications. We have previously demonstrated, for example, that the proteinase inhibitor, PNII undergoes extracellular *in vivo*

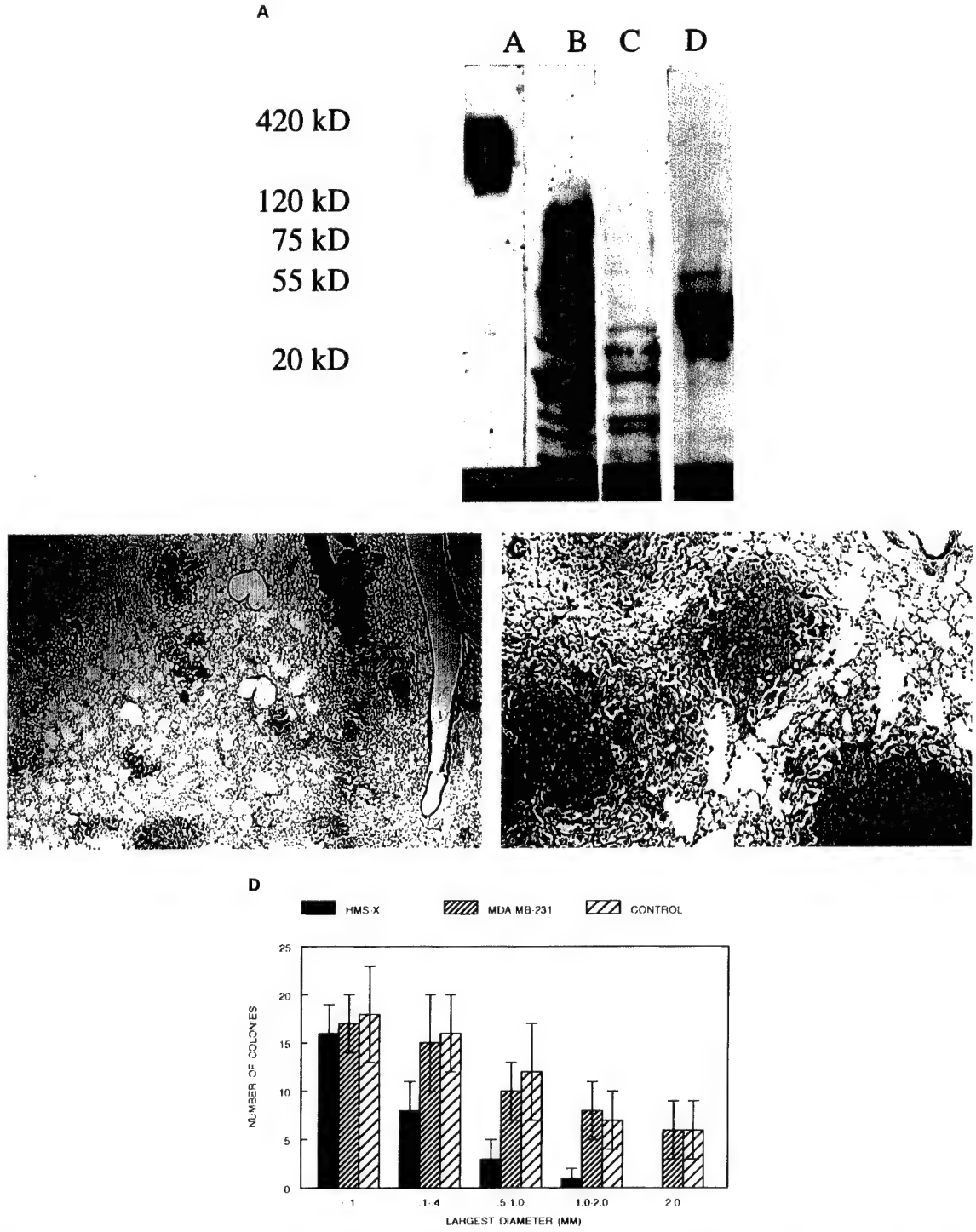


Figure 4 (**a**) Extraction of myoepithelial cell matrix reveals a number of sequestered angiogenic inhibitors and fragments: [A] thrombospondin-1, [B] soluble bFGF receptors, [C] prolactin fragments and [D] plasminogen fragments. (**b**,**c**,**d**) Differences in hematogenous pulmonary metastases with tail vein injected *neo*C8161 is in evidence in mice harboring myoepithelial xenografts (**b**) *versus* non-myoepithelial xenografts (**c**) *versus* control (no xenografts). The number and size of metastatic colonies in mid-longitudinal cross section of lung was determined by digital image analysis and expressed as mean ± standard error. Quantitation of pulmonary metastases revealed similar numbers of colonies in all three groups but a marked decrease in size in the group harboring the myoepithelial xenografts (**d**). Results depict a representative myoepithelial xenograft, HMS-X, a representative non-myoepithelial xenograft, MDA-MB-231, and control (no xenograft). Other myoepithelial and non-myoepithelial xenografts recapitulated these results

processing within the matrices of HMS-X and HMS-4X to a novel 95 kDa fragment retaining full proteinase inhibitor activity and found bound to the myoepithelial matrix (Sternlicht *et al.*, 1996a, 1997). Interestingly when we screened myoepithelial cell CM by Western blots for angiogenic inhibitors, we did not observe evidence of plasminogen or prolactin secretion in the majority of our myoepithelial lines. However when we extracted our myoepithelial xenografts with 2 M urea we found evidence of processed fragments of plasminogen and prolactin in all of our myoepithelial xenografts. Whether these fragments represent angiogenic inhibitors (the 38 kDa angiostatin or the 16 kDa prolactin fragment) (O'Reilly *et al.*, 1994; Clapp *et al.*, 1993) remains to be determined. Since most of our myoepithelial cell lines do not secrete the parental molecules *in vitro*, the fragments are likely derived from circulating murine parental molecules which are sequestered by the anionic myopithelial matrix and processed. There are therefore two types of molecules present within the myoepithelial matrix, molecules secreted by the myoepithelial cells themselves, e.g. thrombospondin-1 and TIMP-1 and molecules sequestered from the circulation, e.g. prolactin and plasminogen and processed. These latter molecules perhaps serve as substrates for unknown enzymes produced by myoepithelial cells. Certain other cell types such as small cell carcinoma can sequester within their stroma, for example, circulating antithrombin and subsequently cleave this molecule to produce a conformationally altered molecule which has antiangiogenic activity (O'Reilly *et al.*, 1999). Myoepithelial cells with their abundant and anionically charged stroma may similarly sequester and process circulating molecules into highly antiangiogenic forms.

Highly metastatic *neo*C8161 cells when injected into the matrices of our myoepithelial xenografts predictably then were not able to stimulate angiogenesis nor metastasize. Since viable *neo*C8161 cells could still be recovered from the xenografts, it likely is the bound angiogenic inhibitors, e.g. thrombospondin-1 and TIMP-1 and possibly the processed fragments of the parental plasminogen and prolactin originally derived from the circulation that are responsible for the local anti-angiogenic effects. Our myoepithelial xenografts were also able to suppress distant pulmonary metastases after hematogenous dissemination of tumor cells following intravenous injection. It is attractive to postulate that this systemic effect on metastasis inhibition is mediated through the release rather than the sequestration of angiogenic inhibitors. One implication then of our *in vivo* findings is that myoepithelial xenografts give rise to circulating inhibitors of angiogenesis. Another possibility to explain our observations is that our myoepithelial xenografts sequester and remove circulating angiogenic factors from serum. Both phenomenon would be anti-angiogenic. We are presently screening serum and urine for antiangiogenic activity and have observed in preliminary studies inhibition of endothelial proliferation *in vitro* with both serum and concentrated urine from mice harboring myoepithelial xenografts. We will be attempting to purify this angiogenic inhibitory activity in the near future.

Past studies have shown that different human and murine cell lines and xenografts produce different angiogenic inhibitors (O'Reilly *et al.*, 1994, 1997, 1999). Myoepithelial cell lines and xenografts exhibit unique anti-angiogenic properties, suggesting that they may represent a natural source of angiogenic inhibitors yet undiscovered.

Materials and methods

Cell lines and xenografts

Informed patient consent and certification from the UCLA Human Subject Protection Committee was obtained. Approval from the Chancellor's Animal Research Committee was also requested and obtained (certification ARC 95-127-11). Early passage (passage 10–15) human myoepithelial cell lines/xenografts: HMS-1-6, X-6X established in our laboratory (Sternlicht *et al.*, 1996a,b, 1997; Shao *et al.*, 1998) were used. Other non-myoepithelial cell lines/xenografts used included the human melanomas, C8161 (Dr Mary Hendrix, University of Iowa) and M15 (Dr Don Morton, John Wayne Cancer Center, Santa Monica, CA, USA); the human breast carcinomas, MDA-MB-231, MDA-MB-468, MCF-7, T47D, BT-549, MDA-MB-157, Hs578T, Hs578Bst; other malignant lines including the A431, vulvar carcinoma, A253, salivary gland carcinoma and HT-29, colon carcinoma (American Type Culture Collection, Rockville, MD, USA). The highly metastatic C8161 line was transfected by us with pSV2*neo* in a previous study (Safarians *et al.*, 1996). Normal cells used included human mammary epithelial cells (HMEC) and human umbilical vein endothelial cells (UVE) (Clonetics, San Diego, CA, USA). Serum free conditioned media (CM) was collected from many of these lines over 24 h and concentrated 10–100-fold using Centriprep-10 concentrators (Amicon, Beverly, MA, USA). Prior to collection of CM, myoepithelial cells were also pretreated with: cyclohexamide (CHX) (40 μg/ml) for 24 h; phorbol 12-myristate 13-acetate (PMA) (5 μM) for 8 h; dexamethasone (0.25 μM) for 24 h. The growth of the different myoepithelial xenografts was observed in 4 week old female athymic/scid mice and compared to the growth of non-myoepithelial xenografts.

Antibodies and probes

High molecular weight genomic DNA was extracted from the myoepithelial and non-myoepithelial xenografts and dot blotted and probed with a murine specific a-^{32}P-dCTP-labeled murine Cot-1 DNA probe (Life Technologies, Inc., Gaithersburg, MD, USA) to determine the murine DNA component of the xenografts (Alpaugh *et al.*, 1999).

Angiogenic factors, angiogenic inhibitors and other relevant molecules were profiled by Western blot analysis of concentrated CM of the cell lines and 2 M urea extracts of the xenografts according to established methods (Sternlicht *et al.*, 1996a, 1997). Western blots were performed as previously described using the manufacturers' recommended dilutions for primary antibodies and a 1 : 50 000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL, USA) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL, USA). Primary rabbit polyclonal antibodies to maspin were obtained (PharMingen, San Diego, CA, USA). Primary antibodies to known angiogenic factors and angiogenic inhibitors included mouse monoclonals or rabbit polyclonals to bFGF, aFGF, angiogenin, TFGα, TGFβ, TNF-α, VEGF, PD-ECGF, PlGF, IFα, HGF, HB-ECGF and IF-α (all from R&D Systems, Minneapolis, MN, USA); plasminogen and PF4 (American Diagnostica, Inc., Greenwich, CT, USA); thrombospondin-1 (Sigma Bio Sciences, St. Louis, MO, USA); rabbit anti-rTIMP-1, (Dr Judith C

Gasson, UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA, USA); prolactin (Dr Richard Weiner, University of California, San Francisco, CA, USA); and bFGF soluble receptor (Dr Anne Hanneken, Scripps Research Institute, LaJolla, CA, USA). Relative levels of expression of the angiogenic factors and angiogenic inhibitors were expressed as 'pie slices'. The pie slices refer to the semiquantitative (relative) levels of expression of a given factor in different (myoepithelial *versus* non-myoepithelial) cell lines. Because of differing affinities of primary antibodies, the size of the pie slices can not be compared among factors. Scion Image software was used for densitometric analyses of the bands and quantification of the signal on Western blots developed by ECL. Also used was a primary rabbit antibody to identify endothelial cells (blood vessels): anti-von Willebrand factor (vWf) (DAKO, Carpinteria, CA, USA). Standardized immunoprecipitation protocols using Sepharose-protein A (Langone, 1982) were employed in the evaluation of thrombospondin-1 activities.

Formalin-fixed paraffin-embedded tissues of human myoepithelial and non-myoepithelial xenografts at a size (1.0 cm diameter) where necrosis was minimal were incubated with rabbit antibodies to vWf (1:1000). Peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody at 1/200 dilution. Colorimetric detection of peroxidase-conjugated secondary antibody was with diaminobenzidine. The number of vWf profiles per high power field (H.P.F.) in 10 high power fields (H.P.F.'s) from each xenograft was determined.

In vitro *angiogenic chemotaxis and proliferation assays*

Standard endothelial migration assays were carried out over 4 h using a chemotaxis chamber (Neuroprobe, Cabin John, MD, USA) with 10 ng/ml bFGF as chemoattractant (Nguyen *et al*., 1993). Effects of HMS-1-6 CM (concentrated 10–100-fold) and HMS-1-6 cells added to the upper compartment were tested. Standard endothelial proliferation assays (O'Reilly *et al*., 1997) were also carried out over 72 h with 25 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA). Heparin-Sepharose NaCl gradient fractionated HMS-1-6 CM was analysed for inhibitory activity.

In vitro *hypoxia studies*

To establish an hypoxic environment (Namiki *et al*., 1995), cells were placed in a Modular Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA), and a pre-analysed air mixture (1% O_2, 5% $C0_2$, and 94% N_2) was flushed through the air-tight chamber for 15 min twice a day. The chamber was then placed in a 37°C incubator, and hypoxia was maintained for 6–24 h. After an initial 6 h period of hypoxia, the cells were harvested and their nuclear fraction collected as follows: Cells were harvested and thawed on ice and reconstituted in 5 volumes of buffer A (10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl) with freshly added supplements (1 M DTT, 0.2 M PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 0.5 M Na_3VO_4). The suspension was then passed through a pre-cooled homogenizer using 25 strokes to lyse the cells. The lysate was then centrifuged for 10 min at 4°C, and the supernatant collected and stored as the cytoplasmic fraction. The pellet was then resuspended in 3.5 volumes of buffer C (0.42 M KCl, 20 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 20% glycerol) with the same supplements as above freshly added. The suspension was then centrifuged for 30 min at 4°C, and the supernatant was collected as the nuclear fraction. Western blot analysis on this nuclear fraction for HIF-1α was performed using a monoclonal antibody to HIF-1α (Lab Vision Corporation, Fremont, CA, USA). After a 24 h period of hypoxia, the cells were harvested, total RNA extracted and 20 μg separated on denaturing agarose gels, transferred to nylon membranes, and probed with a-^{32}P-dCTP-labeled probes using standard Northern blot protocols. cDNA probes used included VEGF (Dr Howard Reber, UCLA) and iNOS (Dr Gautam Chaudhuri, UCLA). The 36P4 probe (Dr Judith Berliner, UCLA) was used as a housekeeping probe.

In vivo *hypoxia studies*

The principle that pimonidazole binds to thiol-containing proteins specifically in hypoxic cells (Varia *et al*., 1998) was exploited by immunocytochemical detection of pimonidazole using a mouse monoclonal antibody (Dr James Raleigh, UNC, Chapel Hill, NC, USA) in tissue sections of the myoepithelial *versus* the non-myoepithelial xenografts grown in mice receiving intraperitoneal injections of pimonidazole hydrochloride (0.5 g/m^2) followed by extirpation of the xenografts 24 h later and immunocytochemical analysis. Pimonidazole immunoreactivity was present as cytoplasmic staining. The number of hypoxic cells (positively stained) was expressed as a percentage of total. Areas showing frank necrosis were also observed and quantitated. Care was taken in these comparative studies to use xenografts of equal size; for these studies xenografts measuring 2.0 cm in diameter were used.

In vivo *angiogenesis and metastasis studies*

The myoepithelial xenografts (HMS-X, HMS-3X and HMS-4X) and non-myoepithelial xenografts (MDA-MB-231, MDA-MB-468) were established in nude and scid mice and allowed to grow to a size of 1.0 cm. This size was chosen because there was no appreciable necrosis in any of the xenografts at this size. 10^5 *neo*C8161 cells suspended in 50λ Hank's Balanced Salt Solution (HBSS) were injected into the centers of the xenografts and allowed to grow for 4–8 weeks. Random xenografts from each group were injected with 50λ trypan blue instead of cells and immediately extirpated to verify that the injections had been delivered to the centers of the xenografts. After 4–8 weeks, animals were sacrificed and primary tumors and lungs were subjected to detailed histopathological examination, collagenase digestion, and culturing of the liberated cells in G418 sulfate (0.2 mg/ml) (Life Technologies, Inc., Gaithersburg, MD, USA). The number of recovered *neo*C8161 clones from each site was determined by phase-contrast image analysis of the cultured dishes. The extirpated xenografts were also studied immunocytochemically for evidence of vWf immunoreactivity and subjected to DNA extraction and murine DNA Cot-1 analysis to estimate the degree of murine angiogenesis. 10^5 *neo*C8161 cells were also suspended in 100λ HBSS and injected into the tail vein (hematogenous metastasis) in mice harboring 2 cm myoepithelial, non-myoepithelial or control (no xenografts). Animals were sacrificed 4 weeks following injection. Removed lungs were inflated, paraffin embedded, sectioned and stained. Both colony number and size (greatest diameter dimension)/mid-longitudinal cross-section of lung were tabulated by digital image analysis, which utilized a Leitz Dialux microscope linked to a Vidicon camera, an IBM PC with PCVision digitizer, and Microscience software. All experiments were performed with groups of 10 mice. Results were analysed with standard tests of statistical significance, including the 2-tailed Student's *t*-test and a one-way analysis of variance (ANOVA).

Abbreviations

UVE, human umbilical vein endothelial cells; CM, conditioned medium; FCS, fetal calf serum; DCIS, ductal carcinoma *in situ*; TIMP-1, tissue inhibitor of metalloproteinase-1; HMEC, human mammary epithelial cells; K-SFM, keratinocyte serum-free medium; vWf, von Will-

ebrand factor; PMA, phorbol 12-myristate 13-acetate; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; TFGα, transforming growth factor α; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; PlGF, placental growth factor; IFα, interferon α; HGF, hepatocyte growth factor; HB-EGF, heparin-binding endothelial growth factor; PF4, platelet factor 4; and iNOS, inducible nitric oxide synthase; HIF-1α, hypoxia inducible factor-1α; HRE, hypoxia response element.

Acknowledgments
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Myoepithelial-Specific CD44 Shedding Contributes to the Anti-invasive and Antiangiogenic Phenotype of Myoepithelial Cells[1]

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Myoepithelial cells surround incipient ductal carcinomas of the breast and exert anti-invasive and antiangiogenic effects *in vitro* through the elaboration of suppressor molecules. This study examines one putative molecule, solubilized CD44 produced by myoepithelial shedding of membrane CD44. Studies with different human myoepithelial cell lines demonstrate that myoepithelial cells express and shed both the 85-kDa standard (CD44s) and the 130-kDa epithelial (CD44v8-10) isoforms, findings further confirmed by the use of isoform-specific antibodies. PMA pretreatment enhances CD44 shedding detected by two different methods at different time points: a reduction in surface CD44 at 2 h by flow cytometry and a marked decrease in both total cellular CD44 and plasma membrane CD44 at 12 h by Western blot. This shedding is both specific for CD44 and specific for myoepithelial cells. This shedding is inhibited by the chymotrypsin inhibitors chymostatin and α_1-antichymotrypsin but not by general metallo-, cysteine, or other serine proteinase inhibitors. Myoepithelial-cell-conditioned medium and affinity-purified solubilized CD44 from this conditioned medium block hyaluronan adhesion and migration of both human carcinoma cell lines and human umbilical vein endothelial cells. © 2000 Academic Press

Key Words: **myoepithelial cells; tumor suppression; CD44; anti-angiogenesis; anti-invasion.**

INTRODUCTION

Myoepithelial cells which surround ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers which arise from this epithelium [1–3]. This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the evolving cancer exists for a number of years only as an *in situ* lesion confined within the ductal system [4]. This *in situ* lesion is commonly termed ductal carcinoma *in situ* or DCIS. Because of their close proximity, myoepithelial cells would be anticipated to exert important paracrine influences on normal, precancerous, and cancerous epithelial cells. Myoepithelial cells of the breast differ from luminal ductal and acinar epithelial cells in many ways: they lack expression of the common hormonal receptor, ER-α, and its responsive genes like PR; they lie next to the basement membrane and contribute to the synthesis of that structure; they rarely transform or proliferate; and when they do give rise to only low-grade benign neoplasms [5, 6]. Myoepithelial cells in a sense can be regarded then as both autocrine and paracrine tumor suppressors. Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5), and bronchus (HMS-6) (with their respective xenografts designated as HMS-X) [1–3, 5, 6]. These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells *in situ* and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α-1 antitrypsin, an unidentified 31- to 33-kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin [1–3]. Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial cell migration and proliferation (angiogenesis) *in vitro* [3, 7]. Our myoepithelial cell lines also inhibit breast carcinoma proliferation *in vitro* through an induction of breast carcinoma cell G_2/M arrest and apoptosis [3], the latter phenomenon of which also occurs *in situ* within DCIS [8].

In our studies demonstrating that myoepithelial cells inhibited both invasion and angiogenesis we noted that treatment with PMA potentiated both types of suppression [1, 7]. In examining possible mecha-

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nisms for these effects we surveyed our myoepithelial cell lines for PMA-induced changes in expression levels of general categories of molecules that had been implicated in tumor invasion and angiogenesis, namely proteinases/proteinase inhibitors, angiogenic factors/angiogenic inhibitors, and adhesion molecules. In the latter category we noted that PMA profoundly decreased the levels of surface CD44 on myoepithelial cells and we decided to study this phenomenon and its significance further in the present study.

MATERIALS AND METHODS

Cell lines. Human myoepithelial cell lines (HMS-1–6) from benign myoepithelial tumors of diverse origins had been previously established in our laboratory [1–3, 5, 6] and maintained in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 μg/ml) and recombinant human epidermal growth factor (5 ng/ml) (GIBCO-BRL, Gaithersburg, MD). Other nonmyoepithelial cell lines used included normal human mammary epithelial cells (HMECs) and normal human umbilical vein endothelial cells (HUVECs) (both from Clonetics, San Diego, CA), the human melanoma C8161 line (a gift of Dr. M. J. C. Hendrix, University of Iowa, Iowa City, IA), and estrogen-receptor-negative breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 (American Type Culture Collection, Rockville, MD). These latter cell lines were maintained in Eagle's MEM supplemented with 10% FBS (GIBCO-BRL) and penicillin–streptomycin antibiotics. Conditioned medium from the myoepithelial cell lines untreated and treated with PMA (5 μM) (Sigma Chemical Co., St. Louis, MO) was collected in basal K-SFM and concentrated up to 10-fold with Centriprep-10 concentrators (Amicon, Beverly, MA).

Pharmacological manipulations. Confluent monolayers of myoepithelial cells were pretreated with tunicamycin (25–200 ng/ml) (Boehringer Mannheim, Indianapolis, IN) for 48 h to study the specific isoforms of CD44 produced by myoepithelial cells. Confluent monolayers of myoepithelial cells (HMS-1-6) and nonmyoepithelial cells (HMECs, C8161, MDA-MB-231, and MDA-MB-468) were also pretreated with PMA (5 μM) (Sigma Chemical Co.) for 20 min followed by harvesting of the cells 1 to 24 h later. The effects of PMA pretreatment were studied in the presence and absence of plasminogen (10 μg/ml) (Sigma Chemical Co.) and in the presence of various proteinase inhibitors. The following specific proteinase inhibitors at a 100-fold range of concentrations (the median concentration so designated) were studied: metalloproteinase inhibitors (0.05 mM EDTA, 3.2 nM TIMP-1, and 0.2 mM 1,10 phenanthroline); serine protease inhibitors (10 μM aprotinin, 0.2 mM leupeptin, 0.2 mM chymostatin and α_1-antichymotrypsin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 nM plasminogen activator inhibitor-1, and 10 nM α_2-antiplasmin); and cysteine proteinase inhibitors (1.0 μM pepstatin A, 10 μM CA-074, 10 μM E-64, and 0.2 mM leupeptin). All inhibitors were obtained from Sigma Chemical Co. except for TIMP-1 (Calbiochem–Novabiochem Corp., San Diego, CA) and CA-074 (Peptides International, Louisville, KY).

Antibodies and probes. Isoform-specific monoclonal antibodies to the standard and epithelial isoforms of CD44 were used. Individual and combinations of MAbs to CD44s (IgG_1-clone DF1485, Zymed Laboratories, San Francisco, CA) and CD44v8-10 (Dr. G. J. Dougherty, UCLA, Los Angeles, CA) were used in immunocytochemical, Western blot, and flow cytometric studies. Other monoclonal antibodies used included anti-E-cadherin (Transduction Laboratories, Lexington, KY) and anti-β_1-integrin (Dr. D. Chang, UCLA). Immunocytochemical studies were performed on formalin-fixed paraffin-embedded sections of human breast tissue according to standard methods. Western blots were performed using the appropriate primary antibodies at the manufacturers' recommended dilutions and a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences). Scion Image software was used for densitometric analysis of bands.

Cellular extractions. Cells were harvested by brief incubation in PBS containing 5 mM EDTA, pelleted, and resuspended in lysis buffer (PBS, 1% (v/v) Nonidet P-40, 5 mM EDTA, and 10 mM PMSF). Lysates were incubated on ice for 10 min and then microcentrifuged for 5 min to pellet nuclei and other insoluble cellular debris. Supernatants were removed and stored at −20°C. Aliquots were thawed, added to an equal volume of nonreducing sample buffer containing 125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8, and incubated at 100°C for 5 min. Total cellular proteins were separated on precast 10% Tris–HCl polyacrylamide electrophoresis gels (Bio-Rad Life Science Products, Hercules, CA) and transferred electrophoretically to nitrocellulose membranes (GIBCO-BRL) and subjected to Western blot analysis. Total RNA was extracted using TriZOL (GIBCO-BRL) and 20 μg of total RNA was loaded/lane in 1.2% agarose gels and Northern blot analyses were performed as previously described [9]. A full-length CD44 cDNA containing the standard CD44 exons (a gift of Dr. G. Dougherty, UCLA) was labeled according to the random primer method of Feinberg and Vogelstein [10]. [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham.

Cell fractionations. PMA-pretreated and untreated cells were homogenized in 20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, 20 μM leupeptin, 0.15 μM pepstatin A at 4°C with a Dounce homogenizer and centrifuged at 13,000*g* for 15 min at 4°C to obtain the particulate fraction. Subcellular fractions of the particulate fraction (P1, nuclear enriched; P2, plasma membrane + mitochondria enriched; and P3, microsomal enriched) were prepared and standard enzyme markers were followed. Further subfractionation of the mitochondria–plasma membrane fraction was carried out by isopycnic centrifugation in Percoll. In some experiments the sedimented membranes were dissolved in SDS sample buffer and assayed for CD44 by Western blot.

Flow cytometric analysis. Myoepithelial cells were pretreated with PMA (5 μM) for 20 min followed by harvesting of the cells 1 to 24 h later. Cells were harvested with PBS containing 5mM EDTA and centrifuged. The pellet was then resuspended with FITC–CD44 mAb or control FITC–IgG and incubated on ice for 30 min. Cells were then washed extensively with HBSS containing 0.1% Na azide and 2% fetal bovine serum and subject to flow cytometry using a Becton Dickinson FACScan.

Purification of shed CD44. A confluent monolayer of HMS-1 was pretreated with PMA (5 μM) for 20 min followed by collection of conditioned medium over the next 24 h. Conditioned medium was collected in basal K-SFM and concentrated up to 10-fold with Centriprep-10 concentrators and subjected to hyaluronan (HA)-affinity chromatography. Purified HA obtained from human umbilical cord was cross-linked to Sepharose 4B. Concentrated HMS-1 conditioned medium (CM) containing shed CD44 was incubated in the affinity column (1 × 15 cm) at 4°C for 15 h. Controls included bovine serum albumin (BSA) cross-linked to Sepharose 4B or cross-linked Sepharose 4B alone. The unbound fraction from the HA affinity column was washed through with 40 ml of 25 mM Tris, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.9% NaCl, pH 7.4. The bound fraction was eluted with 0.2 M glycine HCl, pH 3.5, immediately neutralized with 1.0 M Tris/saline and lyophilized. The lyophilized samples were redissolved in medium and used in HA adhesion and migration assays.

HA adhesion and migration assays. A 24-well plate was coated with HA by adding 500 μl of a solution of potassium hyaluronate purified from human umbilical cord (5 mg/ml in PBS) to each well and incubating the wells overnight at 4°C. Unbound HA was removed by extensive washing with PBS. Nonspecific binding was

blocked by incubating the wells with 10% BSA in PBS for 1 h. The carcinoma cell lines (including the C8161, MDA-MB-231, and MDA-MB-468) and HUVECs (pretreated with bFGF (10 ng/ml)) were separately added to the wells. A total of 2×10^4 cells/well were added in this adhesion assay and incubated for 15 min at 37°C. The effects of HMS-1 CM, anti-CD44, and HA affinity-purified myoepithelial-solubilized CD44 were tested in this adhesion assay by adding these components individually to each well at the time of introducing the cells. Nonadhering cells were removed by a standardized wash procedure. The number of adhering cells was scored using an inverted microscope. Carcinoma cell and endothelial cell migration were separately assayed using a 48-well modified Boyden chamber (Neuro Probe, Inc., Bethesda, MD) with polycarbonate Nucleopore filters of 8 μm pore size (Nucleopore Corp., Pleasanton, CA). The undersides of these filters were precoated with 1 mg/ml HA. Prior to the migration assays, the filters were washed in PBS and air dried. The lower compartments of the modified Boyden chambers were filled with medium containing 10% fetal calf serum (for the carcinoma cell migration assays) or bFGF (10 ng/ml) for the endothelial cell migration assays. A total of 5×10^4 C8161, MDA-MB-231, MDA-MB-468, or HUVECs were separately added to the upper compartments in this migration assay. Chambers were incubated for 6 h at 37°C. After removal of the filters, cells on the noncoated upper membrane side were gently wiped off. Filters were fixed in methanol, stained with Giemsa solution, and mounted on glass slides. Cells that had migrated to the coated undersides of the filters were counted using high-power fields of a light microscope. The effects of HMS-1 CM, anti-CD44, and HA affinity-purified myoepithelial-solubilized CD44 were tested by adding these components individually to each well at the time of introducing the cells. Both the adhesion and migration assays were performed in quadruplicate. Additional controls in both assays included nonmyoepithelial cell CM, murine IgG_1, and the HA affinity column unbound wash through fraction.

Statistical analysis. Results were analyzed with standard tests of statistical significance, including the two-tailed Student's t test and a one-way analysis of variance (ANOVA).

RESULTS

Myoepithelial CD44 Expression

Anti-CD44 immunostaining revealed strong CD44 immunoreactivity in myoepithelial cells surrounding breast ducts and acini (Fig. 1A). All of the myoepithelial cell lines studied (HMS-1–6) expressed both the 85-kDa standard (CD44s) and the 130-kDa epithelial (CD44e) (CD44v8-10) isoforms of CD44. The epithelial isoform was heavily and variably glycosylated producing a smear on Western blot ranging from MW 150–250 kDa. In experiments where HMS cells were pretreated with tunicamycin; however, the heavily glycosylated isoform was reduced to a narrow band of 120–130 kDa, suggesting that it represented the epithelial (CD44v8-10) isoform (Fig. 1B). This fact was confirmed by using epithelial (CD44v8-10) isoform-specific antibodies on Western blot and demonstrating strong signals (data not shown). Epithelial cell lines like HMECs expressed the same CD44 isoforms (the 85-kDa standard (CD44s) and the 130-kDa epithelial (CD44e) (CD44v8-10) isoform) as myoepithelial cells (data not shown).

Myoepithelial-Specific CD44 Shedding

The myoepithelial cell lines studied constitutively shed the 130-kDa epithelial isoform predominantly (Fig. 1C). PMA pretreatment, however, enhanced the shedding of both isoforms ($P < 0.001$) (Fig. 1C). The decrease in cell-associated CD44 and concomitant increase in CD44 in conditioned medium induced by PMA was due to enhanced cell shedding and not a switch in CD44 isoform synthesis from alternative splicing or alterations in levels of CD44 transcripts from changes in gene expression, both of which could contribute to cell surface CD44 alterations (Fig. 1D). Furthermore, the increase in CD44 in conditioned medium never occurred without a concomitant decrease in cell-associated CD44 (Fig. 1C) suggesting that this was a shedding and not a secretory phenomenon. Furthermore, other possibilities to explain the decrease in cell-surface CD44 immunoreactivity such as PMA-induced conformational alterations or PMA-induced alterations in epitope accessibility within the extracellular domain of CD44 were also excluded by these dual observations. Cell fractionation experiments further revealed that virtually all of the CD44 was plasma membrane associated and it was this plasma membrane-associated CD44 that was decreased with PMA pretreatment (Fig. 1E). All of the observations made on Nonidet P-40 detergent extracts of whole cells were confirmed on SDS extracts of purified plasma membranes. These latter studies were necessary because some CD44 activity was retained in the detergent-insoluble pellet of whole cells due to the fact that CD44 is a transmembrane component of partially detergent-insoluble membrane domains, so-called lipid rafts. It was therefore important to use purified plasma membrane preparations to determine what CD44 remained with the plasma membrane and what fraction was truly shed with PMA treatment.

The myoepithelial-cell-associated decrease in CD44 was first in evidence by Western blot 12 h following PMA treatment ($P < 0.001$) (Fig. 2A) but occurred in 2 h by flow cytometric analysis ($P < 0.01$) (Fig. 2B). The PMA-induced reduction in cell-associated CD44 was myoepithelial specific occurring in all of the myoepithelial cell lines examined ($P < 0.001$) but not in any of the nonmyoepithelial cell lines studied ($P > 0.1$) (Figs. 2C, 2D, and 2E). Furthermore, normal HMECs though expressing the same standard and epithelial isoforms of CD44 neither constitutively shed nor shed CD44 in response to PMA (data not shown). Though myoepithelial cells also expressed other adhesion molecules on their surface such as E-cadherin and B1 integrin, they did not shed these molecules constitutively nor in response to PMA ($P > 0.1$) (Fig. 2F). Therefore, the PMA-induced shedding of CD44 was both myoepithelial and CD44 specific. The PMA-induced myoepithelial CD44 shedding was not af-

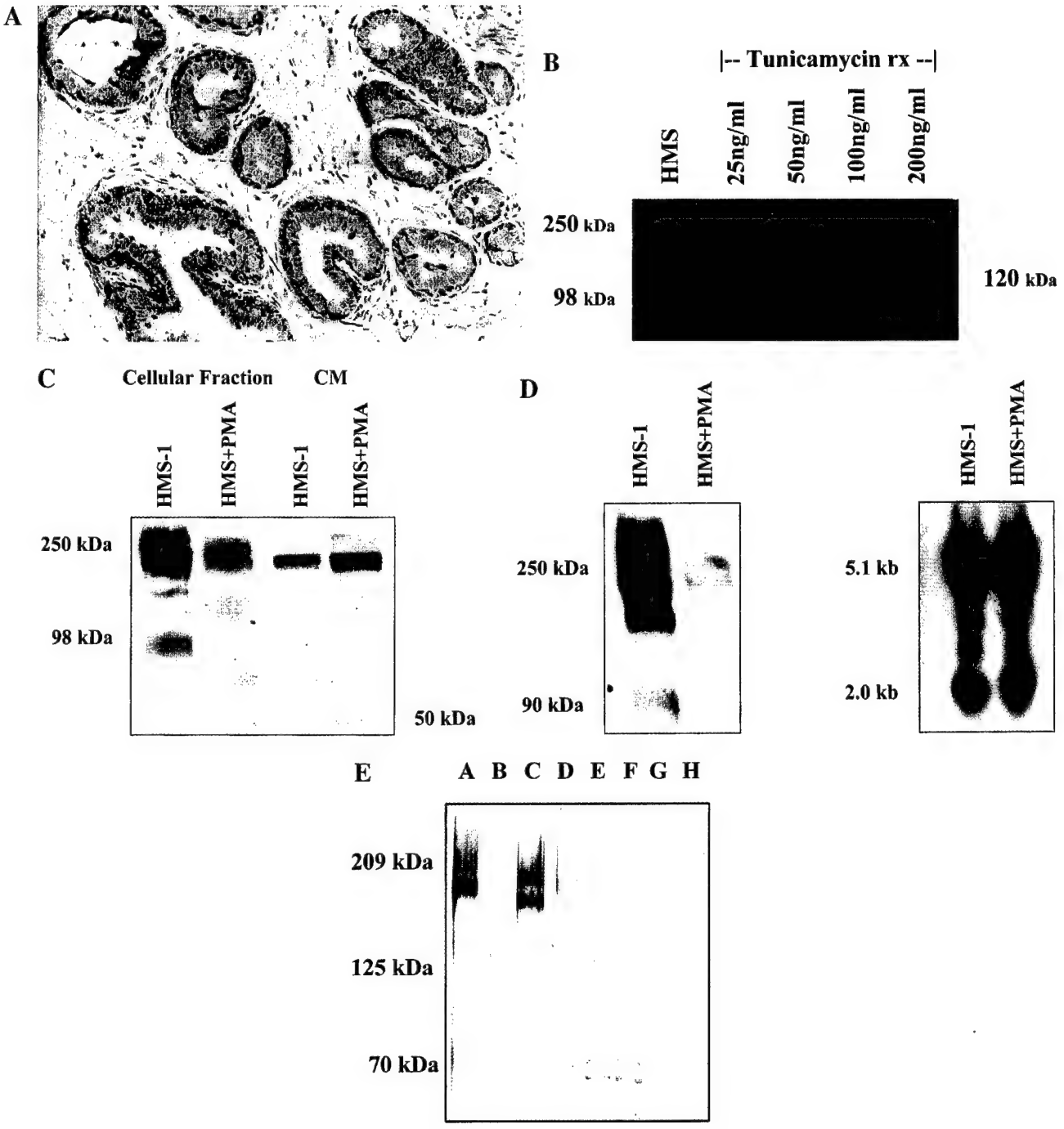

FIG. 1. (A) Anti-CD44 reveals stronger CD44 immunoreactivity in myoepithelial cells surrounding ducts and acini of the breast than in adjacent epithelial cells. In addition to this strong myoepithelial cell CD44 immunoreactivity, scattered CD44 immunoreactivity was also observed at the apical or luminal ends of the ducts and acini (probably reflective of epithelial CD44) as well as within the connective tissue stroma (probably reflective of endothelial cell CD44). CD44 then is a quantitative rather than qualitative marker which distinguishes myoepithelial cells from epithelial and endothelial cells. (B) Tunicamycin at low doses inhibits the synthesis of CD44s and the glycosylation of CD44e, reducing its size to 120–130 kDa and supporting its identity as the epithelial isoform, which was further confirmed by using an epithelial (CD44e) isoform-specific antibody. Tunicamycin at high doses interferes with the synthesis of CD44e as well. (C) Western blot of HMS-1 cellular fractions and CM (concentrated 10-fold) reveals both constitutive and PMA-enhanced CD44 shedding. (D) PMA treatment results in a decrease in cell-associated CD44 (Western blot, left) but no alterations in steady-state mRNA levels nor alternative splicing (Northern blot, right). (E) Cell fractionation experiments with HMS-1 cells reveals that CD44 is present solely in the plasma membrane fraction and the decrease in CD44 following PMA pretreatment is observed in this fraction: lane A, whole cell lysate, no pretreatment; lane B, whole cell lysate, PMA pretreatment; lane C, plasma membrane fraction, no pretreatment; lane D, plasma membrane fraction, PMA pretreatment; lane E, cytosolic fraction, no pretreatment; lane F, cytosolic fraction, PMA pretreatment; lane G, nuclear fraction, no pretreatment; lane H, nuclear fraction, PMA pretreatment.

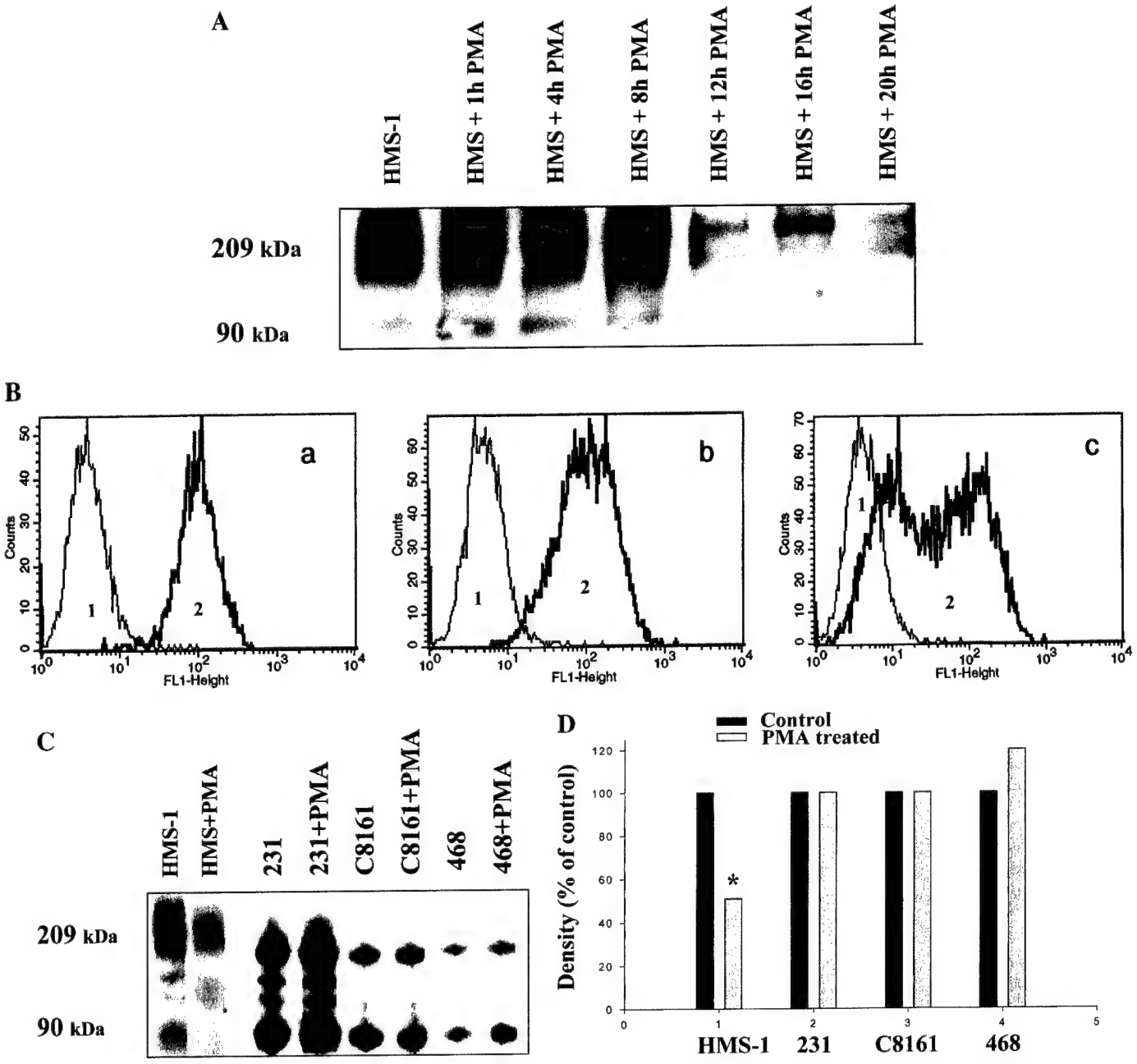

FIG. 2. (A) Time course following 20 min of PMA pretreatment reveals a decrease in cell-associated CD44 at 12 h by Western blot. (B) Flow cytometry reveals a decrease in membrane CD44 at 2 h as demonstrated by an emergence of a cell population with decreased fluorescence. Peak 1, FITC–IgG; Peak 2, FITC–CD44 mAb; a, no treatment; b, PMA pretreatment, harvest at 1 h; c, PMA pretreatment, harvest at 2 h. (C) CD44 shedding induced by PMA is observed only in myoepithelial cells, e.g., HMS-1; other nonmyoepithelial cell lines, MDA-MB-231, C8161, and MDA-MB-468, which express in addition to CD44s alternate isoforms of CD44 (CD44v) not observed in myoepithelial cells, show no shedding of either CD44s or CD44v. (D) Results depicted in (C) are quantitated by densitometric analysis and represent the mean of quadruplicate experiments. CD44 shedding is statistically significant (*) only in myoepithelial cells, e.g., HMS-1. (E) Other myoepithelial cell lines, HMS-3 and HMS-4, exhibit similar shedding. (F) Myoepithelial shedding is limited to CD44; E-cadherin and B1 integrin are not shed. (G) PMA-induced CD44 shedding is inhibited by the chymotrypsin inhibitors chymostatin and α_1-antichymotrypsin but not at all by plasminogen activator inhibitor-1 (PAI-1) or TIMP-1. Other metallo-, cysteine, and serine proteinase inhibitors also do not inhibit CD44 shedding.

fected by either plasminogen addition (10 μg/ml) or depletion ($P > 0.1$) (data not shown). The PMA-induced myoepithelial CD44 shedding could be blocked by the chymotrypsin inhibitors chymostatin and α_1-antichymotrypsin ($P < 0.01$, $P < 0.01$) but not by general metallo-, cysteine, or other serine proteinase inhibitors ($P > 0.1$)

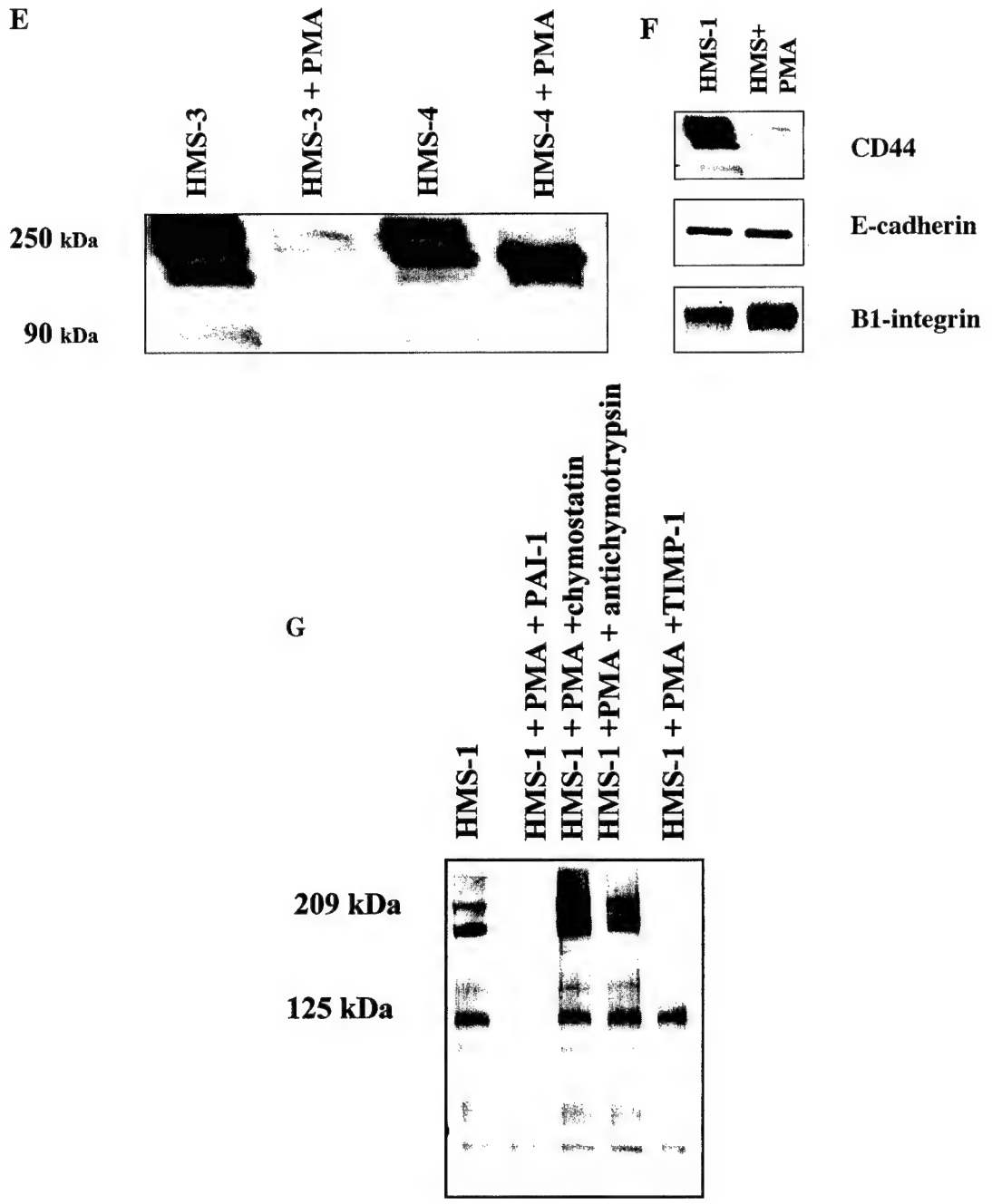

FIG. 2—*Continued*

(Fig. 2G). These results suggested that the mechanism of CD44 shedding involved proteolytic cleavage by a chymotrypsin or chymotrypsin-like protease.

Myoepithelial CD44 Shedding Effects on Carcinoma and Endothelial HA Adhesion and Migration

Myoepithelial cell CM ($P < 0.05$) and affinity-purified myoepithelial-cell-shed CD44 ($P < 0.01$) were effective at inhibiting both carcinoma cell and endothelial cell adhesion and migration involving a HA substrate (Figs. 3A and 3B). Anti-CD44 antibodies ($P < 0.01$) were also effective at inhibiting carcinoma cell and endothelial cell adhesion and migration using this same substrate (Figs. 3A and 3B). These results suggest that both carcinoma cells and endothelial cells utilize their membrane CD44 to adhere to HA and that shed or solubilized CD44 from myoepithelial cells can compete for these HA-binding sites and block the ability of carcinoma and endothelial membrane CD44 to effect its

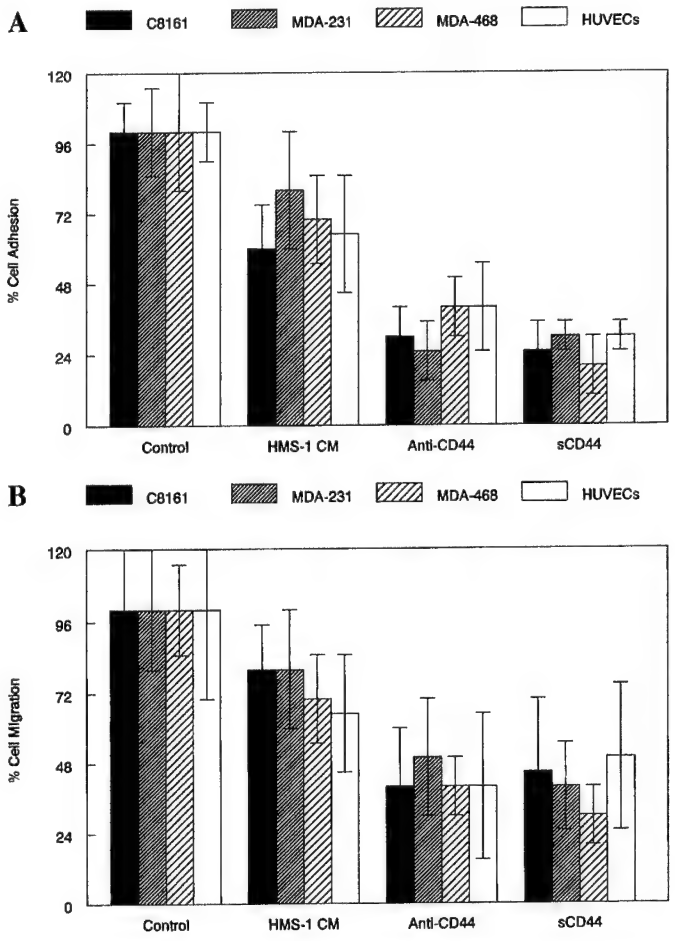

FIG. 3. (A) Effects of HMS-1-conditioned medium (CM) (concentrated 10-fold), anti-CD44 (1/10 dilution), and HA affinity-purified solubilized CD44 (sCD44) (specific binding activity, 5000 ng HA/mg protein) on carcinoma cell (C8161, MDA-MB-231, MDA-MB-468), and HUVEC adhesion on a HA substrate. Baseline cell adhesion for the given cell line is depicted as 100% control and effects of HMS-1 CM, anti-CD44, and purified myoepithelial-solubilized CD44 expressed relative to this control. Results depicted represent means ± standard errors. (B) Effects of HMS-1- conditioned medium (CM) (concentrated 10 fold), anti-CD44 (1/10 dilution), and HA affinity-purified solubilized CD44 (sCD44) (specific binding activity, 5000 ng HA/mg protein) on carcinoma cell (C8161, MDA-MB-231, MDA-MB-468) and HUVEC migration on a HA substrate. Baseline cell migration for the given cell line is depicted as 100% control and effects of HMS-1 CM, anti-CD44, and purified myoepithelial-solubilized CD44 expressed relative to this control. Results depicted represent means ± standard errors.

adhesion and haptotaxis functions. In both the adhesion and the migration assays, the addition of nonmyoepithelial cell CM, murine IgG_1, and the HA affinity column unbound washthrough fraction resulted in no differences from control values (data not shown) ($P > 0.1$).

DISCUSSION

Cancer cells come under the influence of important paracrine regulation from the host microenvironment [11]. Such host regulation may be as great a determinant of a tumor cell's behavior *in vivo* as the specific oncogenic or suppressor alterations occurring within the malignant cell itself and may be mediated by specific extracellular matrix (ECM) molecules, matrix-associated growth factors or host cells themselves [12]. Both positive (fibroblast, myofibroblast, and endothelial cell) and negative (tumor-infiltrating lymphocytes and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior *in vivo* [13, 14]. One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group [1]. Our previous studies have shown that myoepithelial cells and derived cell lines exert multiple suppressive effects on breast carcinoma cells through secretion of a number of different anti-invasive, antiproliferative, and anti-angiogenic molecules [1, 7]. The present study has examined one such molecule, CD44, and specifically its shedding from myoepithelial cells *in vitro* as a potential anti-invasive and antiangiogenic mechanism operating *in vivo*.

CD44 is a cell surface receptor for several ECM components predominant of which is HA but which also include collagen, laminin, fibronectin, and chondroitin sulfate proteoglycan [15]. The binding of HA to CD44 is thought to mediate a number of different biological processes including lymphocytic homing, endothelial chemotaxis (angiogenesis), and tumor cell haptotaxis, invasion and metastasis [16–18]. A number of different regulatory mechanisms exist that can influence the efficacy of CD44–HA binding and the biological processes which are dependent on this interaction. Variant isoforms of CD44 resulting from alternative splicing can influence the affinity of cellular binding [19–24]; phosphorylation of the CD44's cytoplasmic domain can influence the binding properties of its ectodomains [20–24]; and increased shedding or secretion of the CD44 molecule through various mechanisms can either reduce or enhance binding to HA depending on the dynamics of the situation [21–32].

Expression of certain CD44 splice variants are found in several different human cancers. Increased isoform CD44v6 but reduced CD44v9, for example, has been associated with increased metastatic potential [23, 24]. Some studies [25, 26] but not others [27] have implicated specific CD44 isoforms as a prognostic factor in human breast cancer. CD44e (v8-10) is the most common variant isoform in normal epithelial cells and it was this variant that we observed in our myoepithelial cell lines. Significantly greater CD44 immunoreactivity occurred in myoepithelial cells, however. Possible reasons for this included that myoepithelial cells express higher levels of CD44 than their epithelial counterparts or epithelial CD44 is occupied by secreted HA which blocks antibody recognition. Further studies to investigate this latter possibility include preincubating

the sections with hyaluronidase to determine if this changes the pattern of epithelial CD44 staining.

Myoepithelial cells not only express CD44 but shed it constitutively. Since myoepithelial cells rest and synthesize the basement membrane, myoepithelial CD44 may be one adhesion molecule which anchors the myoepithelial cell to the basement membrane. The constitutive shedding of CD44 by myoepithelial cells *in vivo* could therefore participate in normal basement membrane turnover. Our present studies demonstrate that our myoepithelial lines increase their CD44 shedding in response to PMA. This shedding is a true shedding from the plasma membrane and not a secretory phenomenon. The shedding is myoepithelial cell and CD44 specific. Western blot and flow cytometric studies using isoform-specific CD44 antibodies showed that the 130-kDa epithelial isoform was the CD44 isoform that was predominantly shed by myoepithelial cells. Since antibody specificity can rarely be proven to be absolute, and since epitopes are often shared, we, in subsequent studies, have confirmed our findings with PCR using splice-variant-specific primers, given that the entire sequence of the CD44 gene was available. With this more sensitive approach, we have observed that myoepithelial cells also express other CD44 splice variants.

As far as the mechanism of shedding is concerned, increased shedding of ectodomains of molecules such as CD44 could be produced by either extrinsic or intrinsic mechanisms [28]. Hyaluronidase, for example, can digest both HA and the variant isoforms of cell-associated CD44, altering the growth, motility, and metastasizing properties of tumor cells [29]. Alternately CD44 can be cleaved by intrinsic membrane secretases or sheddases of the metalloproteinase class as has been demonstrated recently in certain glioblastoma, pancreatic, and lung carcinoma cell lines [21, 22]. In these malignant cell lines, increased CD44 shedding resulted in increased haptotaxis and migration because it contributed to an autocrine feedback loop of increased CD44 binding to HA. Spontaneous CD44 shedding from murine lymphocytes has also been observed in the circulation of mice to correlate with immune activity and tumor growth [33]. The CD44 shed in this latter setting seemed intact and functional. Our preliminary studies showing inhibition of CD44 shedding in myoepithelial cells by the chymotrypsin inhibitors chymostatin and α_1-antichymotrypsin but not by general metallo-, cysteine, or other serine proteinase inhibitors suggest that the mechanism of CD44 shedding in myoepithelial cells involves proteolytic cleavage by a chymotrypsin or chymotrypsin-like protease. We are currently investigating whether this proteolytic mechanism occurring in myoepithelial cells specifically involves a membrane sheddase. The significance of CD44 shedding on myoepithelial cells would be anticipated to be different from the significance of CD44 shedding on glioblastoma, other malignant cell lines, or lymphocytes since myoepithelial cells are nonmotile and noninvasive [1] and do not migrate even with CD44 shedding. On the other hand soluble CD44 from myoepithelial shedding might be expected to compete with membrane CD44 on carcinoma and endothelial cells for HA-binding sites and our *in vitro* HA adhesion and migration experiments have demonstrated this. Studies by other investigators have also demonstrated that soluble CD44 originating either extrinsically (soluble wild-type CD44–Ig fusion protein) or intrinsically (transfection of cDNAs encoding soluble isoforms of CD44) can compete with tumor cell membrane CD44 for HA-binding sites and exert antitumoral effects including decreased tumorigenicity and increased apoptosis [18, 34, 35]. Since both carcinoma cell invasion and angiogenesis are dependent upon membrane CD44–HA interactions [30–32, 34, 35], myoepithelial-cell-specific shedding of CD44 may reduce the carcinoma and endothelial cell membrane CD44–HA interactions critical to invasion and angiogenesis *in vivo*. Myoepithelial-specific CD44 shedding may therefore contribute to the anti-invasive and antiangiogenic phenotype of myoepithelial cells.

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Myoepithelial-Specific CD44 Shedding Is Mediated by a Putative Chymotrypsin-like Sheddase[1]

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Our previous studies have demonstrated that myoepithelial cells, which surround incipient carcinomas *in situ* of the breast and other organs, exert antiinvasive and antiangiogenic effects *in vitro* through the elaboration of a number of different suppressor molecules among which include the shed membrane CD44. The present study addresses the mechanism of this myoepithelial CD44 shedding. This CD44 shedding is enhanced by PMA pretreatment, is specific for myoepithelial CD44, and inhibited by chymotrypsin-like inhibitors (chymostatin, α_1-antichymotrypsin, TPCK, and SCCA-2) but not by trypsin-like inhibitors (TLCK), nor papain-like inhibitors (SCCA-1) nor hydroxamate-based or general metalloproteinase inhibitors (BB2516 (marimastat), 1,10-phenanthroline, and TIMP-1). The effect of PMA can be mimicked by exogenous chymotrypsin but not by other proteases. The CD44 shedding activity cannot be transferred by conditioned media, cell–cell contact, peripheral membrane, or integral membrane fractions. However, cell-free purified integral plasma membrane fractions obtained from myoepithelial cells pretreated with PMA also exhibit CD44 shedding which is inhibited by chymotrypsin-like inhibitors. These findings support the presence and activation of a putative chymotrypsin-like sheddase as the mechanism of CD44 shedding in myoepithelial cells. © 2000 Academic Press

Key Words: **myoepithelial cells; CD44; chymotrypsin; sheddase; PMA; antiangiogenesis; anti-invasion.**

Abbreviations used: HMS, human matrix-secreting; CM, conditioned media; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of metalloproteinases; PMA, phorbol 12-myristate 13-acetate; DCIS, ductal carcinoma *in situ;* HMEC, human mammary epithelial cells; K-SFM, keratinocyte-serum-free medium; Mab, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DCI, 3,4-dichloroisocoumarin; TLCK, *N* α-*p* -tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-*L*-phenylalanine chloromethyl ketone; SCCA, squamous cell carcinoma antigen; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α; TGFα, transforming growth factor-α; NP-40, Nonidet P-40; PA, plasminogen activator.

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Our previous studies have indicated that myoepithelial cells which surround ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers which arise from this epithelium (1–3). This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the evolving cancer exists for a number of years only as an *in situ* lesion confined within the ductal system (4). Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5) and bronchus (HMS-6) (with their respective xenografts designated as HMS-#X) (1–3, 5, 6). These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells *in situ* and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, α-1 antitrypsin, an unidentified 31-33 kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin (1–3). Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial cell migration and proliferation (angiogenesis) *in vitro* (3, 7). Our myoepithelial cell lines also inhibit breast carcinoma proliferation *in vitro* through an induction of breast carcinoma cell G_2/M arrest and apoptosis (3), the latter phenomenon of which also occurs *in situ* within DCIS (8). In our previous studies demonstrating that myoepithelial cells inhibited both invasion and

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angiogenesis we noted that treatment with PMA potentiated both types of suppression (1, 7). In examining possible mechanisms for these effects we surveyed our myoepithelial cell lines for PMA-induced changes in expression levels of general categories of molecules that had been implicated in tumor invasion and angiogenesis, namely proteinases/proteinase inhibitors, angiogenic factors/angiogenic inhibitors and adhesion molecules. In the latter category we noted in a recent study (9) that PMA profoundly decreased the levels of surface CD44 on myoepithelial cells through a shedding phenomenon. In this study (9), we demonstrated that myoepithelial cells constitutively express and shed both the 85 kDa standard (CD44s) and the 130 kDa epithelial (CD44v8-10) isoforms and that PMA pretreatment enhances this shedding. Our observations were made by using CD44 isoform-specific antibodies as well as CD44 slice-variant-specific primers (9). The decrease in cell-associated CD44 and concomitant increase in CD44 in conditioned media (CM) was due to PMA-enhanced cell shedding and not altered levels of CD44 synthesis from either alternative splicing or changes in gene transcription, either of which could contribute to cell surface CD44 alterations. Furthermore the increase in CD44 in CM never occurred without a concomitant decrease in cell-associated CD44 suggesting that this was a true shedding and not a secretory phenomenon. Furthermore other possibilities to explain the decrease in cell-surface CD44 immunoreactivity such as PMA-induced conformational alterations or PMA-induced alterations in epitope accessibility within the extracellular domain of CD44 were also excluded by these dual observations. Cell fractionation experiments further revealed that virtually all of the CD44 was plasma membrane associated and it was this membrane-associated CD44 that was decreased with PMA pretreatment. The myoepithelial cell-associated decrease in CD44 was first in evidence by Western blot 12 h following PMA treatment but was detected within 2 h by flow cytometric analysis. The PMA-induced reduction in cell-associated CD44 was observed in all 6 of the myoepithelial cell lines examined but not in any of the nonmyoepithelial lines. Furthermore normal mammary epithelial cells (HMECs) though expressing the same standard and variant (epithelial) isoforms of CD44 as myoepithelial cells did not shed CD44 in response to PMA. Because the CD44 shedding phenomenon seemed to be myoepithelial specific, we decided to study it further and investigate its mechanism in the present study.

MATERIALS AND METHODS

Cell lines. Human myoepithelial cell lines (HMS-1–6) from benign myoepithelial tumors of diverse origins had been previously established in our laboratory (1–3, 5, 6) and maintained in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 μg/ml) and recombinant human epidermal growth factor (5 ng/ml). (GIBCO-BRL, Gaithersburg, MD). Other nonmyoepithelial cell lines used included Hs578T (American Type Culture Collection (ATCC), Rockville, MD), and human squamous cell carcinoma lines of salivary gland and vulva, A253 and A431 (ATCC). These latter cell lines were maintained in Eagle MEM supplemented with 10% FBS (GIBCO-BRL) and penicillin–streptomycin antibiotics. Conditioned media from the myoepithelial cell lines untreated and pretreated with PMA (5 μM) was collected in basal K-SFM and concentrated up to 10-fold with Centriprep-10 concentrators (Amicon, Beverly, MA). Coculture experiments involving cell–cell contact of PMA-pretreated and untreated cell types of various combinations were also conducted.

Pharmacological manipulations. Confluent monolayers of myoepithelial cells (HMS-1-6) were also pretreated with PMA (5 μM) (Sigma Chemical Co., St. Louis, MO) for 20 min followed by harvesting of the cells 1 to 24 h later. The effects of PMA pretreatment were studied in the presence and absence of plasminogen (10 μg/ml) and in the presence of various proteinase inhibitors. The following specific proteinase inhibitors at a 10- to 1000-fold range of concentrations (the median concentration so designated) were studied: hydroxamate-based and general metalloproteinase inhibitors including 0.05 mM EDTA, 100 μM TAPI, 3.2 nM TIMP-1, 0.2 mM 1,10 phenanthroline and 100 μM BB2516 (marimastat); trypsin-like, chymotrypsin-like and general serine protease inhibitors including 10 μM aprotinin, 0.2 mM leupeptin, 0.2 mM chymostatin, 0.2 mM α_1-antichymotrypsin, 10 nM plasminogen activator inhibitor–1, 10 nM α_2-antiplasmin, 100 μM 3,4 DCI, 540 nM TLCK, 100 μM TPCK and 5 μM SCCA-2; and papain-like and general cysteine proteinase inhibitors including 1.0 μM pepstatin A, 10 μM CA-074, 10 μM E-64, 10 μM steffin A, 5 μM SCCA-1, 0.2 mM leupeptin and 0.2 mM cystatin. All inhibitors were obtained from Sigma Chemical Co. except for TIMP-1 (Calbiochem-Novabiochem Corp., San Diego, CA), CA-074 (Peptides International, Louisville, KY), BB2516 (a gift of Dr. Howard Reber, UCLA, Los Angeles, CA), SCCA-1 and SCCA-2 (gifts of Dr. Gary Silverman, Children's Hospital, Boston, MA) and TAPI (Immunex Corp., Seattle, WA). Myoepithelial cells (HMS-1–6) and nonmyoepithelial cells (Hs578T, A253, and A431) were also treated with different proteinases. The following proteinases at a 10- to 1000-fold range of concentrations (the median concentration so designated) were used: type I collagenase (human MMP-1) (0.05 mU) (Oncogen, Cambridge, MA), chymotrypsin, trypsin, elastase, pronase (1 U each) (Sigma Chemical Co.), hyaluronidase (1 U) (Sigma Chemical Co.) and cathepsins B (1 U), D (1 U), L (0.5 mU), and G (2.0 mU) (all from Calbiochem, La Jolla, CA).

Antibodies. Monoclonal antibodies to the standard and epithelial isoforms of CD44 were used. Individual and a combination of MAbs to CD44s (IgG_1-clone DF1485, Zymed Laboratories, San Francisco, CA) and CD44v8-10 (Dr. Graeme J. Dougherty, UCLA, Los Angeles, CA) were used in Western blot and flow cytometric studies. Western blots were performed using the appropriate primary antibodies at the manufacturers' recommended dilutions and a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL). A loading control antibody to β-actin (a gift of Dr. Howard Reber, UCLA) was used in all immunoblotting experiments to normalize for cell protein. Scion Image software was used for densitometric analysis of bands. Cells were harvested by brief incubation in PBS containing 5 mM EDTA, pelleted and resuspended in lysis buffer A (PBS, 1% (v/v) NP-40, 5 mM EDTA and 10 mM PMSF). Lysates were incubated on ice for 10 min, then microcentrifuged for 5 min to pellet nuclei and other insoluble cellular debris. Supernatants were removed and stored at −20°C. Aliquots were thawed, added to an equal volume of nonreducing sample buffer containing 125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8, and incubated at 100°C for 5 min. Total cellular proteins were separated on precast 10% Tris–HCl polyacrylamide electrophoresis gels (Bio-Rad Life Science Products, Hercules,

CA), and transferred electrophoretically to nitrocellulose membranes (Gibco BRL, Gaithersburg, MD) and subjected to Western blot analysis.

Flow cytometric analysis. Myoepithelial cells were pretreated with PMA (5 μM) for 20 min followed by harvesting of the cells 1 to 24 h later. Cells were harvested with PBS containing 5 mM EDTA, and centrifuged. The pellet was then resuspended with FITC-CD44 mAb or control FITC-IgG and incubated on ice for 30 min. Cells were then washed extensively with HBSS containing 0.1% Na azide and 2% fetal bovine serum and subject to flow cytometry using a Becton–Dickinson FACScan.

Cell fractionations. PMA-pretreated cells were homogenized in buffer B (20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, 20 μM leupeptin, 0.15 μM pepstatin A) at 4°C with a Dounce homogenizer and centrifuged at 13,000*g* for 15 min at 4°C to obtain the particulate fraction. Subcellular fractions of the particulate fraction (P1, nuclear enriched, P2, plasma membrane + mitochondria enriched; and P3, microsomal enriched) were prepared and recommended enzyme markers were followed as described previously (10). Further subfractionation of the mitochondria–plasma membrane fraction was carried out by isopycnic centrifugation in Percoll. In some experiments the sedimented membranes were dissolved in SDS sample buffer and assayed for CD44 by Western blot. In other experiments the sedimented membranes were subsequently extracted in 1 M NaCl/EDTA (peripheral fraction) or detergent 1% NP-40 (integral fraction) and transferred to a layer of untreated HMS-1 cells to study the effect of this transfer on CD44 shedding. In other experiments the sedimented membranes were suspended in ice-cold Tris–HCl (30 mM, pH 7.2) containing various components to be tested. The reaction mixture (20 μl) was transferred to 37°C and incubated for 1–12 h. The reaction was stopped by the addition of an equal quantity of SDS sample buffer and incubated at 100°C for 5 min. Then aliquots of reaction mixtures were subjected to Western blot analysis using CD44 mAb. The sedimented membranes were also added to 10 volumes of ice-cold 1 M NaCl in 30 mM Tris–HCl (pH 7.2) and kept 15 min on ice. Membranes were collected by centrifugation at 10,000*g* for 15 min at 4°C. Washed membranes were reconstituted in ice-cold Tris–HCl (30 mM, pH 7.2) and incubated and processed as before.

Statistical analysis. Results were analyzed with standard tests of statistical significance, including the two-tailed Student's *t* test and a one-way analysis of variance (ANOVA).

RESULTS

Mechanism of myoepithelial CD44 shedding. The myoepithelial cell-associated decrease in CD44 was detected within 2 h by flow cytometric analysis following PMA pretreatment ($P < 0.01$) (Fig. 1A). The PMA-induced myoepithelial CD44 shedding was not affected by either plasminogen addition (10 μg/ml) or depletion ($P > 0.1$) or α_2-antiplasmin (Fig. 1B), indicating that the shedding was not mediated by plasminogen activators (PA) or plasmin. The PMA-induced myoepithelial CD44 shedding could be blocked however by the chymotrypsin-like serine proteinase inhibitors, chymostatin, α_1-antichymotrypsin, TPCK and SCCA-2 (Fig. 1C) ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.05$) but not by trypsin-like serine proteinase inhibitors like plasminogen activator inhibitor (PAI-1) ($P > 0.1$), papain-like cysteine proteinase inhibitors ($P > 0.1$), or hydroxamate-based or general metalloproteinase inhibitors like TIMP-1 (Fig. 1C) ($P > 0.1$) (Table I). With the chymotrypsin-like proteinase inhibitors, e.g., chymostatin, there was a dose response of inhibition (Fig. 1D). The chymotrypsin-like inhibitors alone in the absence of PMA-pretreatment exerted no effect on myoepithelial CD44. These results implicated a putative chymotrypsin-like sheddase. As further indirect proof, purified exogenous chymotrypsin mimicked the effect of PMA. Chymotrypsin cleaved CD44 on myoepithelial cells over a similar time course of 12 h (Fig. 1E). Chymostatin inhibited the cleavage of CD44 by chymotrypsin. The inhibitory effects of chymostatin and other chymotrypsin-like serine proteinase inhibitors like SCCA-2 (but not the papain-like cysteine proteinase inhibitors like SCCA-1 and cystatin) on both exogenous CD44 cleavage and on PMA-induced myoepithelial CD44 shedding were also observed in flow cytometric studies (Fig. 1F).

Evidence for a specific myoepithelial sheddase. The CD44 shedding activity could not be transferred by CM (Fig. 2A) nor abolished by the removal of CM (Fig. 2B). The susceptibility of myoepithelial CD44 to exogenous proteolytic cleavage was highly chymotrypsin sensitive and relatively insensitive to other proteases (Fig. 2C). Other nonmyoepithelial CD44 did not exhibit this chymotrypsin sensitivity (Fig. 2D). These other nonmyoepithelial CD44 also did not exhibit PMA-induced shedding (data not shown). Coculture experiments where PMA-pretreated HMS-1 cells were subsequently mixed with untreated HMS-1 cells at dilutions to insure cell–cell contact between each of the populations showed no evidence of CD44 shedding in the untreated cells; furthermore when peripheral membrane fractions or integral membrane fractions of PMA-pretreated HMS-1 cells were transferred to untreated HMS-1 cells there was no evidence of CD44 shedding in the HMS-1 cells (data not shown). However cell-free membrane preparations of PMA-pretreated HMS-1 cells showed a progressive CD44 shedding over 1–8 h (Fig. 2E). This CD44 shedding was equally observed in membranes that had been washed with 1 M NaCl in 30 mM Tris–HCl (pH 7.2) to remove peripheral membrane proteins suggesting that the factor responsible for the CD44 shedding was an integral membrane protein. As before, this CD44 shedding could be inhibited by the chymotrypsin-like serine proteinase inhibitors but not by the trypsin-like serine proteinase inhibitors, the papain-like cysteine proteinase inhibitors or the metalloproteinase inhibitors. CD44 shedding was not observed in membrane preparations obtained from HMS-1 cells that were initially untreated or subsequently treated with proteinase inhibitors alone. The collective findings suggest that the membrane factor responsible for shedding and its substrate, CD44, both have to be present in *cis* orientation for activity and support the presence and activation of a putative

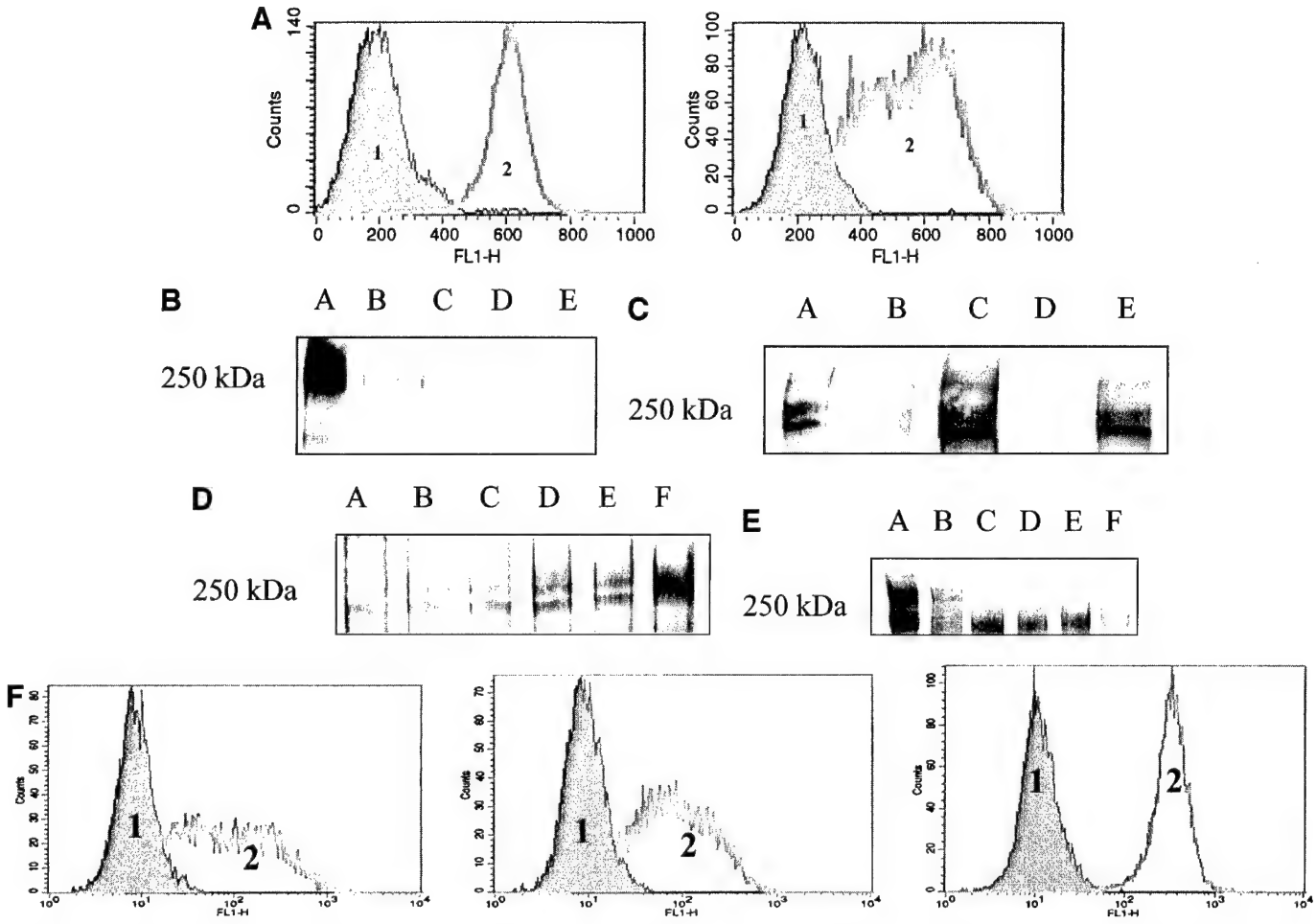

FIG. 1. CD44 flow cytometry or Western blot of HMS-1 cells: (A) Flow cytometry of untreated HMS-1 cells depicts cell surface CD44 fluorescence (top). PMA pretreatment results in a decrease in cell surface CD44 at 2 h as demonstrated by an emergence of a cell population with decreased fluorescence (bottom). Peak 1, FITC-IgG; peak 2, FITC-CD44 mAb; top, no pretreatment; bottom, PMA pretreatment. (B) PMA-induced CD44 shedding is not affected by either the presence or absence of plasminogen or the presence of α_2 antiplasmin: Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1; lane C, PMA-pretreated HMS-1 cells in the absence of plasminogen; lane D, PMA-pretreated HMS-1 cells in the presence of plasminogen; lane E, PMA-treated cells in the presence of α_2 antiplasmin. (C) PMA-induced CD44 shedding is inhibited by chymostatin and α_1-antichymotrypsin but not at all by plasminogen activator inhibitor-1 (PAI-1) or TIMP-1. Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1 in the presence of PAI-1; lane C, PMA-pretreated HMS-1 in the presence of chymostatin; D, PMA-pretreated HMS-1 cells in the presence of TIMP-1; E, PMA-pretreated HMS-1 cells in the presence of α_1-antichymotrypsin. Other chymotrypsin-like serine proteinase inhibitors, TPCK, and SCCA-2 (Table I) were effective at inhibition but not inhibitors belonging to other classes. (D) Dose response of increasing concentrations of chymostatin on CD44 shedding inhibition: Lane A, PMA-pretreated HMS-1 cells in the presence of 2 μM chymostatin; lane B, PMA-pretreated HMS-1 in the presence of 10 μM chymostatin; lane C, PMA-pretreated HMS-1 in the presence of 20 μM chymostatin; lane D, PMA-pretreated HMS-1 in the presence of 200 μM chymostatin; lane E, PMA-pretreated HMS-1 in the presence of 1 mM chymostatin; lane F, PMA-pretreated HMS-1 in the presence of 2 mM chymostatin. Other chymotrypsin-like inhibitors showed a similar dose response. (E) Time course of exogenous chymotrypsin (1 unit) cleavage of HMS-1 CD44. Lane A, untreated HMS-1 cells; lane B, HMS-1 cells treated with chymotrypsin (1 unit) for 1 h; lane C, HMS-1 cells treated with chymotrypsin (1 unit) for 2 h; lane D, HMS-1 cells treated with chymotrypsin (1 unit) for 4 h; lane E, HMS-1 cells treated with chymotrypsin (1 unit) for 8 h; lane F, HMS-1 cells treated with chymotrypsin (1 unit) for 12 h. (F) Flow cytometry of PMA-pretreated HMS-1 cells in the presence of cystatin, top; PMA-pretreated HMS-1 cells in the presence of SCCA-1, middle; PMA-pretreated HMS-1 cells in the presence of SCCA-2, bottom. Only SCCA-2 inhibited the CD44 shedding. Peak 1, FITC-IgG; Peak 2, FITC-CD44 mAb.

chymotrypsin-like sheddase as the mechanism of PMA-induced CD44 shedding in myoepithelial cells.

DISCUSSION

Cancer cells come under the influence of important paracrine regulation from the host microenvironment (11). Both positive (fibroblast, myofibroblast and endothelial cell) and negative (tumor infiltrating lymphocytes and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior *in vivo* (12–14). One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group (1). Our previous studies have shown that myoepithelial

TABLE I

Representative Inhibitory Spectrum of the Putative Myoepithelial CD44 Sheddase

Proteinase	Inhibitory class	Activity
EDTA	M	-----
TIMP-1	M	-----
1,10-Phenanthroline	M	-----
BB2516	M	-----
TAPI	M	-----
PAI-1	S	-----
3,4 DCI	S	-----
TLCK	S	-----
TPCK	S	+++
SCCA-2	S	+++
α-1-Antichymotrypsin	S	+++
Steffin A	C	-----
SCCA-1	C	-----
Chymostatin	S, C	++++
CA-074	C	-----
E-64	C	-----
Cystatin	C	-----

Note. M, metalloproteinase inhibitor; S, serine proteinase inhibitor; C, cysteine proteinase inhibitor.

cells and derived cell lines exert multiple suppressive effects on carcinoma cells through secretion of a number of different anti-invasive, antiproliferative, and antiangiogenic molecules (1, 7). Another candidate paracrine suppressor molecules is shed CD44. The present study has examined the mechanism of myoepithelial CD44 shedding.

CD44 is a cell surface receptor for several extracellular matrix components predominant of which is hyaluronan but which also include collagen, laminin, fibronectin and chondroitin sulfate proteoglycan (15). The binding of hyaluronan to CD44 is thought to mediate a number of different biological processes including lymphocytic homing, endothelial chemotaxis (angiogenesis) and tumor cell haptotaxis, invasion and metastasis (16–18). A number of different regulatory mechanisms exist which can influence the efficacy of CD44–hyaluronan binding and the biological processes which are dependent on this interaction. Variant isoforms of CD44 resulting from alternative splicing can influence the affinity of cellular binding (19–24); phosphorylation of the CD44's cytoplasmic domain can influence the binding properties of its ectodomains (20–24); and increased shedding or secretion of the CD44 molecule through various mechanisms can either reduce or enhance binding to hyaluronan depending on the specifics and the dynamics of the situation (21–29).

Our present studies demonstrate that myoepithelial cells shed CD44. The shedding is myoepithelial cell CD44 specific. Increased shedding of ectodomains of molecules such as CD44 can be produced by either extrinsic or intrinsic mechanisms (21, 22, 25–28). Hyaluronidase, for example, can digest both hyaluronan and the variant isoforms of cell-associated CD44, altering the growth, motility and metastasizing properties of tumor cells (25, 26). Alternately CD44 can be cleaved by intrinsic membrane secretases or sheddases (21, 22).

Our studies addressing the mechanism of the PMA-induced CD44 shedding in myoepithelial cells indicate that a putative chymotrypsin-like sheddase is involved. There has been a recent interest in membrane sheddases or membrane convertases as they are sometimes designated (30–41). Membrane sheddases have been implicated in the shedding of a number of different membrane and cell surface molecules which include a diverse range of membrane proteins of Type I or Type II topologies (31). Examples of molecules shed by sheddase mechanisms include Alzheimer's amyloid precursor protein, angiotensin converting enzyme, TGF-α, the tumor necrosis ligand and receptor superfamilies (33) and cell adhesion molecules such as L-selectin (38, 39) and CD44 (21, 22, 36, 37). Most of the sheddases identified to date have been metalloproteinases but not necessarily matrix metalloproteinases (33, 38, 39). The identification of these sheddases has rested mainly on indirect evidence as ours has, which is based on the specificities of a broad spectrum of proteinase inhibitors. The vast majority of putative sheddases have not been purified or cloned. The one exception has been tumor necrosis factor-α converting enzyme (TACE), a metalloproteinase-disintegrin sheddase, demonstrated to have catalytic function, and thought responsible for the shedding of diverse cell surface proteins including, in addition to TNF-α, TGFα and L-selectin but interestingly not CD44 (35). CD44 has been reported to be cleaved and shed, on the other hand, by stimulated human granulocytes and certain malignant cell lines including glioblastoma lines. Both cellular sheddings were induced by PMA and inhibited by metalloproteinase inhibitors including TIMP-1 and 1,10-phenanthroline (21, 22, 36, 37). In our studies with myoepithelial cells, diverse metalloproteinase inhibitors including TIMP-1, 1,10-phenanthroline, EDTA, and hydroxamate-based inhibitors, TAPI and BB2516 were not effective in inhibiting PMA-induced CD44 shedding; rather diverse chymotrypsin-like serine proteinase inhibitors but not trypsin-like serine or papain-like cysteine proteinase inhibitors were effective. When we first began investigating the inhibitory spectrum of our putative sheddase, we began with general inhibitors of each proteinase class. We initially noted that chymostatin exhibited the greatest inhibitory activity. Because this proteinase inhibitor had general inhibitory activity against both chymotrypsin-like serine proteinases as well as cysteine proteases, we further defined the inhibitory spectrum of our putative sheddase by investigating two additional proteinase inhibitors: cystatin and α_1-antichymotrypsin.

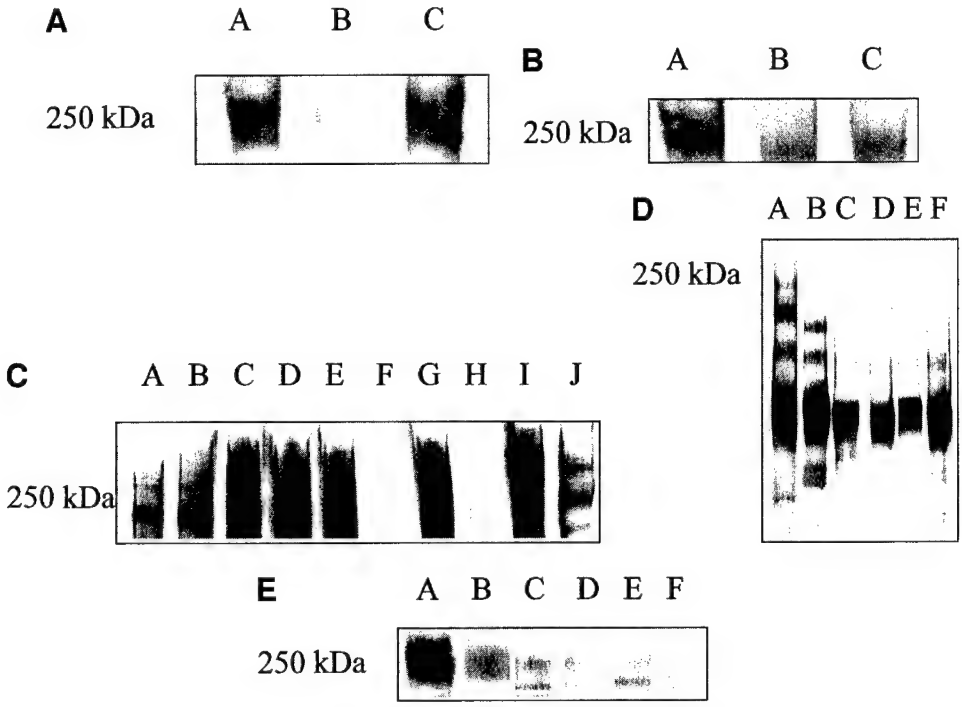

FIG. 2. Western blots of HMS-1 fractions: (A) HMS-1 CD44 sheds following PMA pretreatment but this activity cannot be transferred in CM: Lane A, untreated HMS-1 cell; lane B, PMA-pretreated HMS-1 cells; lane C, CM from HMS-1 cells pretreated with PMA and transferred to untreated HMS-1 cells followed by the usual cellular harvest. (B) HMS-1 CD44 sheds following PMA pretreatment but this activity can not be abolished by hourly removal of CM over the ensuing 24 h from HMS-1 cells following PMA-pretreatment. Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1 cells; lane C, HMS-1 cells pretreated with PMA whose CM was removed hourly for 24 h followed by the usual cellular harvest. (C) Susceptibility of myoepithelial CD44 to different proteases: Cathepsin G (lane A); cathepsin L (lane B); cathepsin D (lane C); cathepsin B (lane D); trypsin (lane E); chymotrypsin (lane F); hyaluronidase (lane G); elastase (lane H); MMP-1 (lane I); pronase (lane J). No enzyme other than chymotrypsin and elastase cleaved myoepithelial CD44. (D) Exogenous chymotrypsin CD44 cleavage is specific for myoepithelial cells: Lane A, untreated Hs578T cells; lane B, chymotrypsin-treated Hs578T cells; lane C, untreated A253 cells; lane D, chymotrypsin-treated A253 cells; lane E, untreated A431 cells; lane F, chymotrypsin-treated A431 cells. Although these nonmyoepithelial cells express alternate forms of CD44, their CD44 does not exhibit chymotrypsin cleavage. (E) CD44 shedding in membrane preparations of PMA-pretreated HMS-1 cells. A progressive decrease in CD44 is observed over time: Lane A, no pretreatment; lane B, PMA pretreatment followed by 1 h incubation; lane C, PMA pretreatment followed by 2 h incubation; lane D, PMA pretreatment followed by 3 h incubation; lane E, PMA pretreatment followed by 4 h incubation; lane F, PMA pretreatment followed by 8 h incubation.

The former had inhibitory activity against papain-like cysteine proteases where the latter had inhibitory activity against serine proteinase inhibitors. We observed inhibitory activity only with α_1-antichymotrypsin. Then we compared SCCA-2 with SCCA-1. These latter inhibitors are proteinase inhibitors that map to a serpin cluster at 18q21.3 but differ in inhibitory spectrum (42, 43). SCCA-1 inhibits only papain-like cysteine proteases, whereas SCC2-A inhibits chymotrypsin-like serine proteinases but interestingly not purified chymotrypsin (42, 43). Yet SCCA-2 was active against our putative myoepithelial sheddase. Finally we compared TPCK, an inhibitor of chymotrypsin-like serine proteinases with TLCK, an inhibitor of trypsin-like serine proteinases and found that only TPCK was active against our putative myoepithelial sheddase. These four separate lines of evidence, although indirect, all point to a putative chymotrypsin-like sheddase.

From our studies, three requirements were necessary for CD44 shedding to occur (1): The substrate, CD44, had to be susceptible to cleavage in the first place. The susceptibility of a particular isoform of CD44 to cleavage may not necessarily be a function of its primary amino acid sequence determined by its specific splice variation but rather a function of its tertiary structure and/or degree of glycosylation. CD44 molecules on nonmyoepithelial cells were not susceptible to cleavage by exogenous purified chymotrypsin suggesting that their CD44 might not be susceptible to shedding from an endogenous chymotrypsin-like sheddase even if one existed in those cells (2). There must be activation of the sheddase. In our past studies, we have shown that PMA activates and causes membrane association of protein kinase C (44). Presumably this association activates the sheddase responsible for CD44 shedding. The mechanism of activation remains unknown but could be due to direct activation of the

sheddase by phosphorylation, increased access to the substrate as a result of phosphorylation, or conformational modification of the substrate itself making it susceptible to cleavage. From our studies we can not yet determine whether nonmyoepithelial cell lines lack the sheddase or lack the activating response to PMA or both since their CD44 does not appear to be susceptible to chymotrypsin cleavage in the first place (3). Even if the CD44 is of the type which is susceptible to cleavage and even if the membrane sheddase exists and can be activated, both sheddase and CD44 substrate must be in *cis* orientation. Actually this requirement is confirming evidence that our putative chymotrypsin-like activity is indeed a sheddase. Since the sheddase is not secreted, transfer of activity or abolishment of activity by removal of CM could not be achieved. Transfer of sheddase activity likewise could not be achieved by cell–cell contact or membrane extracts because both produce a *trans* orientation.

Our observations that myoepithelial cell CD44 is equally susceptible to both exogenous chymotrypsin cleavage as well as endogenous PMA-induced shedding is again indirect evidence that we are dealing with a chymotrypsin-like sheddase. Certainly we do not know whether both exogenous and endogenous molecules cleave the same sites of CD44, only that CD44 is susceptible to cleavage by both molecules. Obviously the exogenous chymotrypsin is not restricted by the *cis* orientation requirement.

The significance of CD44 shedding from myoepithelial cells would be anticipated to have paracrine tumor suppressive effects on both carcinoma cells themselves as well as on endothelial cells (angiogenesis) from recent studies (45, 46). These studies have demonstrated that soluble CD44 can have autocrine suppressive effects on tumor cells: soluble CD44 originating either extrinsically (soluble wild-type CD44–Ig fusion protein) or intrinsically (transfection of cDNAs encoding soluble isoforms of CD44) can compete with tumor cell membrane CD44 for hyaluronan binding sites and exert antitumoral effects including decreased tumorigenicity and increased apoptosis (18, 45, 46). Since both carcinoma cell invasion and angiogenesis are dependent upon membrane CD44-hyaluronan interactions (27–29, 45, 46), myoepithelial cell specific shedding of CD44 could reduce the carcinoma and endothelial cell CD44-hyaluronan interactions critical to invasion and angiogenesis *in vivo.* CD44 shedding from myoepithelial cells would therefore be anticipated to have paracrine suppressive effects on tumors.

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Fiberoptic Ductoscopy for Patients with Nipple Discharge

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BACKGROUND. Breast carcinoma and precancer are thought to start in the lining of the milk duct or lobule, yet until recently, we have not had direct access to this area other than by blindly removing tissue by core biopsy or fine-needle aspiration. Fiberoptic ductoscopy (FDS) is an emerging technique allowing direct visual access to the ductal system of the breast through nipple orifice exploration.

METHODS. We applied ductoscopy to 259 women who had nipple discharge, and we analyzed the visual findings, the cytological washings, and the subsequent histopathology.

RESULTS. In 92 (36%) of these women, fiberoptic ductoscopy was successful in detecting an intraductal papillary lesion. Of these observed lesions, 68 (74%) were single papilloma, 21 (23%) were multiple discrete papillomas, and 3 (3%) were diffuse intraductal thickening which corresponded to diffuse papillomatosis on histopathological analysis. The overall positive predictive value of FDS screening was 83%. Of the lesions observed, 29.8% were located in the main (segmental) duct, 43.9% lesions in the first branch, 17.5% lesions in the second branch, 7.9% in the third branch, and 0.9% in the fourth branch. These lesions had an overall average distance of 2.7 cm from the nipple orifice. Ductal washings performed at the time of ductoscopy were effective at obtaining representative exfoliated ductal cells which could be evaluated for the presence of clumps (> 50 cells), clumps with atypia or single ductal cells. The presence of clumps with positive FDS increased the positive predictive value to 86%.

CONCLUSIONS. Fiberoptic ductoscopy currently offers a safe alternative to ductography in guiding subsequent breast surgery in the treatment of nipple discharge. *Cancer* **2000;89:1512–9.** *© 2000 American Cancer Society.*

KEYWORDS: nipple discharge, intraductal papillary lesions, fiberoptic ductoscopy.

Breast cancer and precancer are thought to start in the lining of the milk duct or lobule, yet until recently, we have not had direct access to this area other than by blindly removing tissue by core biopsy or fine-needle aspiration. Fiberoptic ductoscopy is an emerging technique allowing direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration.[1]

Spontaneous nipple discharge is a common health problem reported to occur at least intermittently in up to 10% of women who undergo routine health examinations.[2] In this article, discharge refers to any type of discharge which can be serous, serosanguineous, milky, watery, or bloody. Common causes of these various types of nipple discharge are intraductal papilloma or papillomatosis, which are observed in 35% to 48% of cases based on surgical pathology analysis of excised tissues.[3] Because of this, surgery is indicated when the diagnosis is suspected.[2] Because many cases of nipple discharge occur, however, without the presence of an intraductal lesion, breast duc-

tography has been a technique employed in the United States to indicate the presence and location of a site of intraductal obstruction thought to correspond to the presence of intraductal papilloma. Breast ductoscopy, however, is a technique which historically has not enjoyed popularity in the United States. Whereas early attempts at ductoscopy used a rigid ductoscope, fiberoptic ductoscopy is now an emerging technique allowing direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration. This newer technique has seen isolated and limited use in the United States in pilot studies of two groups of women: women with nipple discharge and high risk women without discharge.[1] The current study was designed to examine the efficacy of fiberoptic ductoscopy (FDS) and ductal lavage in a larger series of 259 women, all with nipple discharge, to determine whether fiberoptic ductoscopy might offer a safe alternative to ductography in precisely diagnosing intraductal papillary lesions and guiding subsequent breast surgery.

METHODS

Patients

Informed patient consent and certification from the Institutional Human Subject Protection Committee of the Cancer Hospital, Shanghai Medical University, was obtained prior to all studies. Between October 1997 and December 1998, 259 female patients with nipple discharge gave their concent and were enrolled in the study. These patients received fiberoptic ductoscopy, ductal lavage, and subsequent breast surgery when indicated.

Fiberoptic Ductoscopy System (FDS)

FDS (FVS-3000, Fujikura Co., Ltd., Tokyo, Japan) consists of a silicafiberscope, a light source, an image monitor, and an image recorder, which can video record or directly photograph the observed image. An outer air channel of the fiberscope permits the installation and irrigation of saline washings and retrieval of cells from the ductal system of the breast. The outer diameter of the silicafiberscope we used was 0.72 mm and its maximum exploratory length was 6.5 cm. In select patients, we also used a double barrel lumen catheter designed to maximize ductal lavage.

FDS Procedure

The nipple and areola of the breast were cleaned with 70% ethanol and Povidone-iodine (Betadine) disinfectant. Bowmann's lacrimal dilators with outer diameters of 0.35 mm and 0.45 mm, respectively, were lubricated with xylocaine jelly and then inserted into the discharging nipple orifice to dilate the ostium of the lactiferous duct. The fiberscope then was inserted into the duct orifice. About 10 ml of normal saline then was perfused into the duct through the air channel of the fiberscope to insure the patency of the duct during the procedure. The lactiferous duct, lactiferous sinus, and the segmental duct and its branches were observed in orderly succession. The presence and appearances of any papillary lesions were noted, and the transductal distances from the nipple orifice to the proximal and distal borders of the lesions were measured and recorded.

Ductal Lavage and Cytology

The fiberscope was retracted and the instilled saline was retrieved and processed for cytology. A double lumen ductoscope was inserted in selected patients where the retrieved instilled saline was limited, and ductal washings were obtained by irrigation and processed for cytology. Cytological analysis consisted of standard cytospin preparations and Papanicolaou and Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL) staining. The cytological findings could be grouped into three categories: clumps of ductal cells (> 50 cells), clumps with atypia, and single ductal cells or small clumps.

Pathological Analysis

Patients then underwent breast surgery. Detailed histopathologic analysis of the extirpated tissues was carried out to evaluate the intraductal abnormalities present.

Statistical Analysis

Standard tests of significance were carried out. These included comparisons of differences among variables with the Student's t test for paired data and the Spearman rank based correlation to assess the relation between the variables. The log rank test was used to assess the univariate effect of certain variables on the presence or absence of an intraductal lesion. The Cox proportional hazards model was used to assess these effects after adjustment for other covariates.

RESULTS

FDS Screenings

Of the 259 patients studied, 92 (36%) were observed to have an intraductal papillary lesion (solitary, multiple, or diffuse) by FDS examination. In 167 (64%) patients, the FDS screening was negative. All of the 92 FDS positive patients had ductal lavage at the time of ductoscopy with cytological analysis and detailed histopathological examination of their subsequently re-

TABLE 1
Value of FDS Screening in Women with Nipple Discharge

	+Histopathology	−Histopathology
Ductoscopy results	Number of patients	Number of patients
+FDS (n = 92)	76	16
−FDS (n = 65)	12	53

Specificity 77%; Sensitivity 88%; Positive Predictive Value 83%; Negative Predictive Value 82%.

sected breast tissue specimen. All of the 167 FDS negative patients had ductal lavage, but only 65 elected to have surgery. Therefore, detailed histopathologic follow-up was available in only a subset of these FDS negative patients. Of the 92 patients with a positive FDS screening, 76 (83%) patients had intraductal papillary alterations (either solitary papilloma, multiple papilloma, or diffuse papillomatosis) on histopathological analysis, but 16 (17%) patients contained no such papillary alterations. Of the 65 patients with a negative FDS examination, only 12 (18%) had papillary alterations on histopathologic examination of the excised tissues. In half of these latter cases, the papillary alterations were small intraductal papillomas involving small to medium sized ducts probably beyond the fourth branch point of the ductal system, and, in the other half, there was diffuse papillomatosis of small ducts. For nipple discharge diagnosis, FDS screening exhibited high sensitivity and high specificity in detecting intraductal papillary lesions as well as high positive and high negative predictive values (Table 1).

The vast majority of women (> 95%) with nipple discharge have open ducts, so insertion of the ductoscope caused them no discomfort or pain. Therefore no anaesthesia was necessary. For the < 5% of women who were apprehensive, we applied a xylocaine jelly solution to the areola and nipple also. These patients also reported no discomfort or pain, and their initial apprehension faded during the procedure. The FDS technique should not be considered invasive. The flexible ductoscope entered patients' breasts but did not transgress the myoepithelial layer of the ductal system. We observed no untoward effects or complications in any of the 259 patients examined by FDS screening. Specifically, patients reported no persistent pain or discomfort from the procedure; we observed no mastitis, subareolar inflammation, or bleeding as a result of the procedure; and we saw no increase in nipple discharge following the procedure. Therefore, there was a high degree of patient acceptance and compliance with the technique and an absence of possible complications.

TABLE 2
Clinical Characteristics of Patients with Positive vs. Negative FDS Screening

	+FDS		−FDS	
Clinical characteristics	No. patients	Percentage	No. patients	Percentage
Menopausal status	92	100.0	167	100.0
Pre	68	73.9	125	74.8
Post	19	20.7	31	18.6
Castration	5	5.4	11	6.6
History (total)	92	100.0	167	100.0
Papilloma	4	4.4	5	3.0
Breast cancer	2	2.2	7	4.2
Negative	86	93.4	155	92.8
Localization-discharge	92	100.0	167	100.0
Unilateral	90	97.8	163	97.6
Bilateral	2	2.2	4	2.4
No. of discharge ducts	92	100.0	167	100.0
Single	88	95.7	160	95.8
Multiple	4	4.3	7	4.2
Duration of discharge	92	100.0	167	100.0
1 month	7	7.6	18	10.8
2–3 months	42	45.7	74	44.3
4–12 months	21	22.8	36	21.6
> 12 months	22	23.9	39	23.3
Discharge characteristic	92	100.0	167	100.0
Bloody[a]	30	32.6	31	18.6
Serosanguineous	16	17.4	38	22.7
Serous	42	45.6	87	52.1
Milky	2	2.2	6	3.6
Watery	2	2.2	5	3.0
Association with mass	92	100.0	167	100.0
No	80	87.0	140	83.8
Yes	12	13.0	27	16.2

[a] Significant by univariate analysis, $P = 0.01$; insignificant in multivariate analysis, $P = 0.27$, when compared to FDS with respect to histopathology.

Clinical Characteristics of the Patients in the Study

The mean age of all patients was 46 years (range 20–75 years). Before FDS screening, the average duration of nipple discharge had been 20 months and the longest duration had been 20 years. The clinical characteristics of the patients are shown in Table 2. Except for the presence of bloody discharge which was seen more frequently in the positive FDS group ($P = 0.01$ by univariate analysis), there were no statistically significant differences in any of the clinical parameters which distinguished the positive from the negative FDS group. In multivariate analysis, bloody discharge compared with FDS screening was less significant ($P = 0.027$) as a predictive marker for the presence of a histopathologically confirmed intraductal papillary lesion.

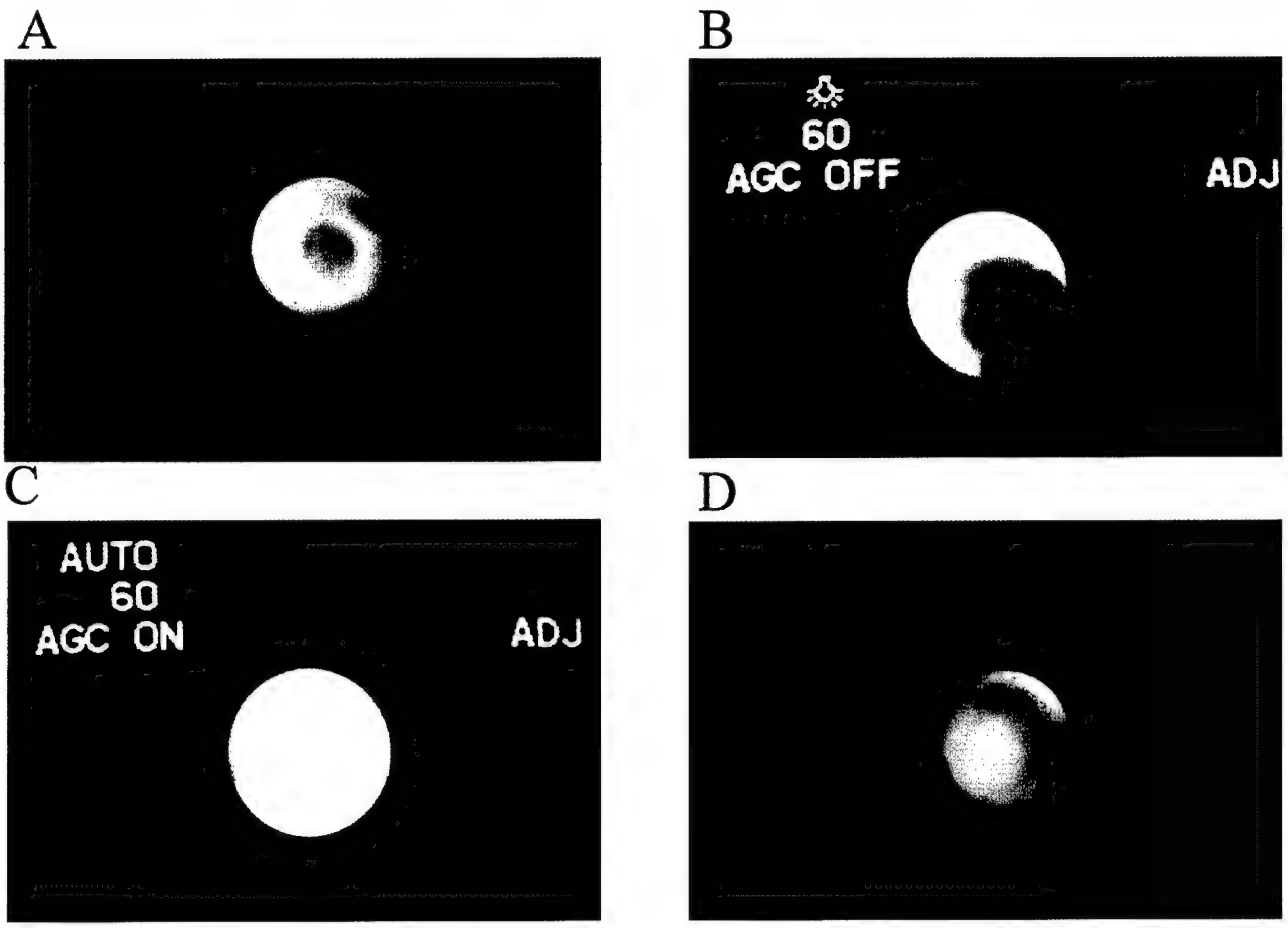

FIGURE 1. (A) Appearance of a normal segmental duct at the first bifurcation where two lumens are present; (B) Immediately after the first bifurcation of the same case, a large reddish intraductal proliferation representing a solitary intraductal papilloma was observed; (C) In a different case, a large intraductal proliferation was present as a rounded white mass; (D) In yet another case the intraductal papilloma was present as an obstructing intraluminal mass with a thin rim of patency still present at 12–4 o'clock. The proximal and distal margins of the papilloma could be measured in terms of transductal distances from the nipple orifice and used to guide subsequent surgery.

The Ductoscopy Technique, Instrumentation, and Analysis

The learning curve for cannulation of the ducts, advancing the ductoscope, and observing the lesion was fairly steep with incremental gains in skill seen during the first 10 cases. After that point, the examiner reached a steady state of maximum versatility with the technique. It should be pointed out that the specialists who performed the technique were surgeons with basic skills in the use of instrumentation of this sort. The cost of the equipment was $400,000 (U.S. dollars) for the silicafiberscope, the light source, the image monitor, and the image recorder. Each fiberscope could be reused for 100 patients. The cost of a fiberscope replacement was $1000 (U.S. dollars).

Experienced pathologists and cytologists could learn easily how to interpret cells obtained by ductal lavage, but as with any new technique, they first must learn the different cytological features that were present in cells obtained by ductal lavage and apply these newly learned criteria. The cells obtained were somewhat different in morphology and far greater in number than cells present in routine nipple aspirates.

Observation of the Breast Ducts with FDS

Normal ductal cavities presented appearances ranging from lustrous pale yellow to pink and were observed to exhibit ring folds on the duct walls. The main segmental duct could readily be observed to bifurcate into primary branches (Figure 1A). The appearance of an intraductal papillary lesion under FDS examination was either red, yellow, or ash-gray. The lesion appeared as a polypoid mass projecting into the lumen of the duct (Figures 1B, 1C, 1D). The majority of intraductal papillary lesions were present either as sol-

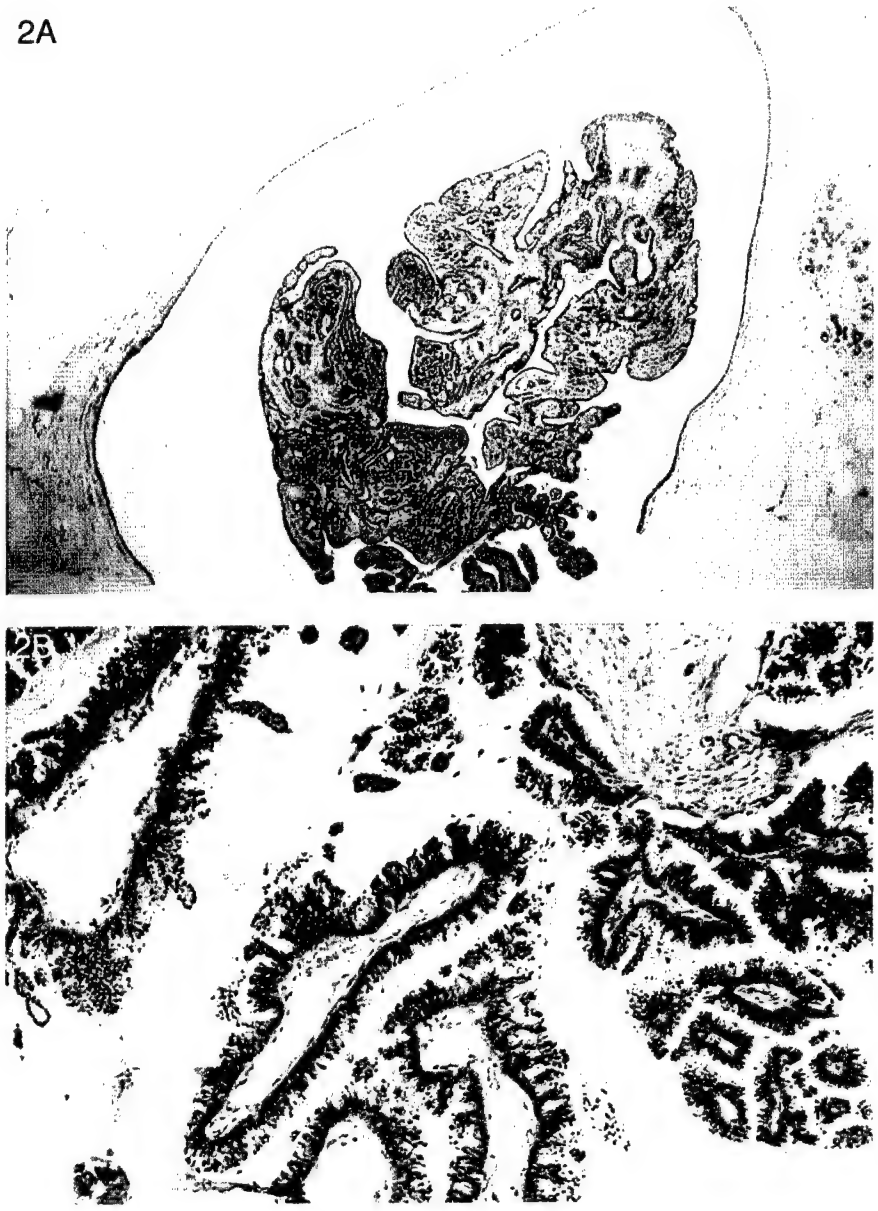

FIGURE 2. (A) Appearance of the classic solitary intraductal papilloma depicted in Figure 1B is demonstrated by histopathology of the resected specimen; (B) four (4.5%) of the intraductal papillary lesions in the study exhibited areas of atypia or areas of frank carcinoma; the lesion depicted represented an atypical intraductal papillary proliferation that was part of an extensive intraductal papillary carcinoma from a case where there also was adjacent invasive carcinoma. (C) This atypical papillary proliferation gave rise to abnormal cytology in ductal lavage.

itary lesions (Figure 2A) or multiple lesions in a single ductal system (Table 3), but a very small minority occurred in two different ductal systems, either unilaterally or bilaterally (Table 2). In 3% of the positive FDS patients, no discrete intraductal papillary lesion was observed, but rather a diffuse ductal thickening was noted. The presence of this diffuse intraductal thickening corresponded to the finding of diffuse papillomatosis on histopathologic study.

The shortest transductal distance from intraductal papillary lesion to the nipple orifice was 0.5 cm, the longest was 6 cm, and the average was 2.7 cm. The transductal distances to the proximal and distal margins of the intraductal lesions were recorded and used along with intraductal catheters at the time of surgical excision. The intraductal lesions occurred in the main segmental duct and the first, second, third, and fourth branches in decreasing frequency (Table 4).

FIGURE 2. (*continued*)

TABLE 3
Numeric Distribution of Lesions in Patients with Positive FDS

Number of lesions	Number of patients	Percentage (%)
One	68	74.0
Two	15	16.3
Three	6	6.5
Diffuse	3	3.2
Total	92	100.0

TABLE 4
Geographic Distribution of Lesions in Patients with Positive FDS

Location	Number of lesions	Percentage (%)
Segmental	34	29.8
First branch	50	43.9
Second branch	20	17.5
Third branch	9	7.9
Fourth branch	1	0.9
Total	114	100.0

TABLE 5
Value of Cytology in Positive vs. Negative FDS Screening

	Overall total (n = 157) no. of patients		+FDS (n = 92) no. of patients		−FDS (n = 65) no. of patients	
Cytology	+Path	−Path	+Path	−Path	+Path	−Path
Clumps > 50 cells	35	15	32	6	3	9
Atypical clumps	4	0	4	0	0	0
Single cells	52	51	45	5	7	46
Specificity	77%		45%		84%	
Sensitivity	43%		44%		30%	
Positive Predictive Value	72%		86%[a]		25%	
Negative Predictive Value	50%		10%		87%[a]	

[a] Value greater than FDS alone

Cytological Analysis of Ductal Lavage

Cytological findings were grouped into three categories: clumps of ductal cells (> 50 cells), clumps with atypia (based on nuclear pleomorphism, chromatin staining, and size), and single ductal cells or small clumps. For the purposes of this study, we assumed that large ductal clumps reflected the exfoliation of an intraductal papillary lesion and that single ductal cells reflected the absence of the same. The positive and negative predictive values of cytologic analysis alone for the presence/absence of an intraductal papillary lesion were 72% and 50%, respectively (Table 5). These cytologic values were less than the corresponding predictive values of FDS which were 83% and 82%, respectively (Table 1). However, when cytologic analysis was combined with FDS, the positive predictive value of the combination was 86% and the negative predictive value was 87% (Table 5). Therefore cytologic analysis combined with FDS screening provides the best preoperative assessment of the likelihood that an intraductal papillary lesion is the cause of nipple discharge.

All four cases in the positive FDS group that exhibited atypical clumps of ductal cells were associated with intraductal papillary lesions showing atypical features: either areas of atypical ductal hyperplasia (two cases) or ductal carcinoma in situ (one case), or frankly intraductal papillary carcinoma (one case) (Figure 2B). Therefore the presence of atypical clumps (Figure 2C) together with a positive FDS screening exhibited a 100% positive predictive value for an atypical intraductal papillary lesion.

DISCUSSION

This study demonstrates the value of FDS and ductal lavage cytology as a screening and preoperative procedure in women with nipple discharge. Certainly clinical characteristics do not distinguish the patients whose nipple discharge is due to intraductal papillary lesions from those due to other causes. Furthermore, clinical characteristics do not distinguish the type of papillary lesion, its singularity, multiplicity, or its degree of atypism. FDS and ductal lavage cytology do.

FDS is gaining in popularity as thinner, longer, and more flexible fiberoptic scopes become available to permit better observation of the ductal system of the breast. Breast ductoscopy has evolved slowly over the past decade. In 1988, through a rigid endoscope of 1.7 mm in its outer diameter, Teboul[4] was first to succeed in observing the duct cavity under the guidance of ultrasound. In 1989, Makita[5] established blind intraductal biopsy of the breast for pathologic diagnosis of nipple discharge through a rigid endoscope of 1.25 mm in its outer diameter. The endoscope that he used was similar to an arthroendoscope. Later Okasaki,[6] in cooperation with the Fujikura Company, developed a breast fiberoptic ductoscopy system making it possible to observe directly the duct cavities and duct walls and to project them onto a screen. This technique allowed for observation of even minute intraductal lesions.

In patients with nipple discharge, many are found not to have an intraductal papillary lesion. In most studies, intraductal papilloma and papillomatosis account for, at most, 35%–48% of cases of nipple discharge. By papillomatosis, we mean a diffuse multicentric hyperplasia involving major ducts. We use the term papillomatosis because many of the lesions were papillary in appearance, but we acknowledge that this papillomatosis is a form of epithelial hyperplasia. In our study, only 92 of 259 (36%) patients with nipple discharge had demonstrable intraductal papillary lesions by FDS screening. Breast duct endoscopy, therefore, can screen these patients effectively. If it finds no demonstrable lesion, patients may be spared surgery or may choose single duct ligation rather than excision. Conversely, positive FDS would suggest surgery and would dictate the type and extent of surgery. A variety of clinicopathologic subtypes of papillary lesions, which include solitary and multiple papillomas, diffuse papillomatosis, papillomas with atypical ductal hyperplasia or focal DCIS, and papillary carcinomas have been observed in the ductal system.[7,8,9] All of these subtypes were observed by FDS in our study and sampled by ductal lavage. FDS showing multiple papillomas or diffuse papillomatosis suggests a more extensive duct excision than FDS showing a single intraductal papilloma. FDS with atypical cytology suggests a more extensive resection of subareolar tissues since the presence of atypical ductal hyperplasia, focal DCIS or frank intraductal papillary carcinoma merits wider excision to achieve completely negative surgical margins.

The ductal system of the breast illustrates a significant degree of branching morphogenesis. Intraductal papillary lesions can exist in any branch. Since the direct observation of intraductal lesions is limited by two ductoscope parameters, its length of 6.5 cm and its outer diameter of 0.72 mm (which limits access to ducts smaller than this diameter), it is conceivable that peripheral intraductal lesions causing nipple discharge are beyond the reach of the ductoscope and could be missed. In fact, for the 12 patients in our study who had a negative FDS screening but papillary lesions on histopathologic examination, their histopathologic analysis revealed either small papillomas in small to medium sized ducts, ducts probably beyond the fourth branch or diffuse papillomatosis in small ducts. This peripheral distribution of some intraductal papillary lesions probably explains the FDS examination false negative rate of 18%. As longer, thinner, and more flexible probes become available, this false negative rate likely will decrease.

Intraductal papillary lesions are usually minute lesions growing in the ductal wall. They are fragile and can easily exfoliate. This may explain the difficulty in finding papillary lesions in surgical specimens on gross examination and the absence of suspected papillary lesions on histopathologic examinations of resected specimens. This fragility and minuteness might explain, in part, why 17% of patients with positive FDS had no demonstrable lesion on histopathologic examination. Conceivably, in some of these patients ductoscopy itself could have exfoliated the lesion so that no lesion remained when histological analysis was carried out. Therefore, the 17% false positive rate may be considerably lower.

Although this study did not compare ductoscopy with ductography specifically, it seems intuitive that ductoscopy provides advantages over ductography in

TABLE 6
Historical Comparisons of Breast Ductoscopy with Breast Ductography

Feature	Ductoscopy	Ductography
Proximal limits of lesion	Yes	Yes
Distal limits of lesion	Yes	No
Duct obstruction required	No	Yes
Direct observation	Yes	No
Precise distances	Yes	No
Cytologic analysis	Yes	No

the routine management and examination of patients with nipple discharge. Ductoscopy allows direct observation of lesions and not just indirect shadowing. Ductoscopy, unlike ductography, allows more precise localization and mapping of the lesion within the duct. This mapping is not dependent on ductal obstruction and includes both proximal and distal margins of the lesion and not just the former. Ductoscopy, unlike ductography, allows intraductal sampling of the ductal cells for cytologic analysis which in turn increases both positive and negative predictive values. The historical advantages of ductoscopy over ductography are summarized in Table 6. In terms of specificity, sensitivity, positive and negative predictive values in detecting an intraductal lesion in patients with nipple discharge, we are currently conducting a prospective randomized trial comparing ductoscopy with ductography. However before conducting and reporting on such a comparative study, we felt it important to report first the positive results with ductoscopy itself.

The unique advantages of ductoscopy should result in its ability to direct and limit subsequent breast surgery more precisely, so that surgery can remove the offending lesion and yet spare as much uninvolved breast tissue as possible.

Breast ductoscopy is very inexpensive by present standards. In mainland China, it costs only $50 U.S. dollars. In the United States, it probably would cost approximately $200. Certainly this screening technique is no more expensive than ductography and only slightly more expensive than nipple smear aspiration cytology.

Ductoscopy in the future may permit direct intraductal ablation of the lesion by either mechanical or chemical means which would spare surgery altogether in selected patients. The benefit of intraductal ablation certainly would be cost effective when compared to surgery.

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chemotherapy. A phase II trial has been started to assess the therapeutic efficacy of this combination.

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Breast-cancer diagnosis with nipple fluid bFGF

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Early diagnosis of breast cancer is the key to extending survival of breast-cancer patients. We found that the concentrations of nipple fluid bFGF (basic fibroblast growth factor) was significantly increased in breast-cancer patients compared with concentrations in controls (1717 ng/L [SD 706] *vs* 19 ng/L [19]; Student's *t* test p=0·027). Measurement of bFGF in nipple fluid could be a useful diagnostic tool for breast cancer, and deserves further study.

Breast cancer is the most frequently diagnosed cancer in American women. The key to extending survival is early diagnosis. Early detection through screening mammography saves lives; however, mammography fails to detect 20% of breast cancers. Breast biopsy samples from women with abnormal mammograms confirm cancer in only 10–20% of cases. Furthermore, current serum tumour markers are not useful in diagnosing breast cancer.[1]

The process of angiogenesis has a critical role in breast tumour growth and metastasis. Studies from our laboratory and from other institutions have shown that angiogenic factors can be significantly increased in the serum and urine of breast-cancer patients.[2] The concentration of bFGF (basic fibroblast growth factor) has been shown to correlate with the disease stage of the tumour.[3] However, it cannot be used as a screening tool because there is significant overlap between normal individuals and cancer patients. We assessed whether the concentration of these angiogenic peptides in the nipple fluid, which have half lives measured in min, can be used as a diagnostic tool for breast cancer. Because breast cancer arises from the epithelium of the terminal duct lobular unit, we expected that the fluid secreted into the breast ducts would contain a much higher concentration of these angiogenic factors than serum or urine.

We measured bFGF by ELISA (R&D Systems, Minneapolis, USA) in the nipple fluid from ten patients without benign breast conditions (controls), four lactating women, and ten women with stage 1 or 2 breast cancer (table). We were able to obtain nipple fluid from these 24 women but attempted to get samples from 30 women (80% success rate). This study was approved by the UCLA Institutional Review Board, and all participants signed informed consent forms. The detection limit was 100 ng/L. Nipple fluid from controls had significantly lower concentrations of bFGF compared with nipple fluid from cancer patients (19 ng/L [SD 19] *vs* 1717 ng/L [706]; p=0·027 Student's *t* test). There was very little overlap in the values of bFGF measured in these two groups. The one cancer patient with undetectable concentrations of bFGF in the nipple fluid had already had surgical resection of the cancer at the time of this study. Lactating nipple fluids contained a significant amount of bFGF (1065 ng/L [251]).

We also measured the concentrations of another potent angiogenic factor—VEGF (vascular endothelial growth factor)—in nipple fluids, and found no significant differences between the three groups: 106 500 ng/L [19 000] in controls; 92 400 ng/L [19 100] in women with cancer; and 46 100 [17 800] ng/L in women who were lactating. We conclude that VEGF in nipple fluids would not be useful in diagnosing breast cancer.

Patients' nipple fluid has not been extensively investigated as a possible source for diagnostic purposes. Previous studies with carcinoembryonic antigen and prostate specific antigen in nipple fluid showed significant overlap between the study groups.[4,5] Measurement of bFGF in nipple fluid has the potential to be a useful diagnostic tool for breast cancer. Our results are preliminary, and a larger study is warranted to test this hypothesis.

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Controls	Lactating	Breast cancer
190	1670	7470
ND	1150	2490
ND	990	2390
ND	450	2240
ND	..	860
ND	..	670
ND	..	590
ND	..	310
ND	..	150
ND	..	ND

ND=not detected.

Nipple fluid bFGF (ng/L)

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Surgical Endoscopy
Ultrasound and
Interventional Techniques

Fiberoptic ductoscopy for breast cancer patients with nipple discharge

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Abstract
Background: Breast cancer and precancer are thought to originate in the lining of the milk duct, but until recently, we have not had direct access to this area other than in tissue removed blindly by core biopsy or fine-needle aspiration. Fiberoptic ductoscopy (FDS) is an emerging technique that allows direct visual access of the ductal system of the breast through nipple orifice cannulation and exploration. To date, this technique has been used only in pilot studies. Previously, we have demonstrated that fiberoptic ductoscopy in patients with and without nipple discharge is a safe and effective means of visualizing the intraductal lesion. When combined with cytology, it is a screening technique that has high predictive value.
Methods: We applied ductoscopy to 415 women with nipple discharge with the specific intent of detecting those patients with nipple discharge who had intraductal carcinoma (DCIS) as the basis of their discharge.
Results: In this cohort of patients, ductoscopy was successful in visualizing an intraductal lesion in 166 patients (40%). In these cases, ductal lavage following ductoscopy increased the yield of cytologically interpretable ductal epithelial cells 100-fold compared to discharge fluid alone. In the majority of these patients, FDS examination detected lesions that had the appearance of typical papillomas. However, in 10 patients, the intraductal lesion exhibited one of several atypical features, including bleeding, circumferential obstruction, and gross fungating projections. In eight of these patients, the subsequent histopathology turned out to be DCIS. In two of these eight patients, endoscopic biopsy revealed cytologically malignant cells; in two others, ductal lavage (washings) revealed cytologically malignant cells. In three additional patients, although FDS examination uncovered a typical papilloma that was not biopsied, ductal lavage (washings) revealed cytologically malignant cells. On surgical pathology review of the extirpated lesions, all 11 patients were subsequently shown to have DCIS. Of these 11 cases of DCIS that were initially detected with a combination of FDS and ductal lavage cytology, six were completely negative on mammogram and physical exam.
Conclusion: Although nipple discharge is an unusual presentation for DCIS, in patients with nipple discharge, FDS with ductal lavage cytology is a useful technique for diagnosing DCIS prior to definitive surgery.

Key words: Nipple discharge — Breast cancer — Fiberoptic ductoscopy — Intraductal carcinoma — Cancer

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Breast cancer and precancer are thought to originate in the lining of the milk duct or lobule. Yet until recently we have not had direct access to this area other than by examining tissue removed at biopsy. Fiberoptic ductoscopy (FDS) is an emerging technique that allows direct visual access to the ductal system of the breast through nipple orifice cannulation and exploration [3, 7]. In previous studies [3, 8], we have used FDS successfully in women with and without nipple discharge.

Although nipple discharge is relatively common and usually benign in origin, it can in fact herald the onset of intraductal carcinoma of the breast (DCIS). The incidence of DCIS in patients with nipple discharge varies from 1% to 10% [5]. The diagnostic workup of patients with nipple discharge usually includes clinical history, physical examination, mammography, ductography, and nipple discharge cytology but not FDS [2]. In a previous study, we demonstrated that in patients with nipple discharge, FDS is a safe and effective means of visualizing the intraductal lesion. Moreover, when combined with ductal lavage cytology, it is a screening technique that has a high predictive value [8].

In the present study, we applied ductoscopy to 415 women with nipple discharge with the specific intent of detecting those patients whose nipple discharge was the result of DCIS.

Materials and methods

Patients

Informed patient consent and certification from the Institutional Human Subject Protection Committee of the Cancer Hospital, Shanghai Medical University, was obtained prior to all studies. Between October 1997 and March 2000, 415 patients with nipple discharge consented and were enrolled in the study. These patients were subjected to FDS, endoscopic biopsy when indicated, ductal lavage with cytological analysis, and subsequent surgery when appropriate.

Fiberoptic ductoscopy system (FDS)

The FDS setup (FVS-3000; Fujikura Co. Ltd., Tokyo, Japan) consisted of a silica fiberscope, a light source, an image monitor, and an image recorder, which was capable of videorecording or directly photographing the observed image. An outer air channel of the fiberscope allowed for the installation and irrigation of saline washings and the retrieval of cells from the ductal system of the breast. The outer diameter of the silica fiberscope was 0.72 mm; its maximal exploratory length was 6.5 cm. In select patients, a double-barrel lumen catheter designed to maximize ductal lavage was also used.

FDS procedure

The nipple and areola of the breast were cleaned with 70% ethanol and povidone-iodine disinfectant (Betadine). Bowmann's lacrimal dilators with outer diameters of 0.35 mm and 0.45 mm, respectively, were inserted sequentially into the discharging nipple orifice to dilate the ostium of the lactiferous duct. The fiberscope was then inserted into the duct orifice.

The procedure is not at all uncomfortable for the patients. When the patients have nipple discharge, their nipple orifice is already somewhat dilated and easily accessible. The majority of patients required no anesthesia at all; a minority required only the local lubrication of the nipple orifices with xylocaine jelly. No other forms of local or general anesthesia were used. The patients were not given IV sedation. This is a painless procedure.

About 10 ml of normal saline was then perfused into the duct through the air channel of the fiberscope to ensure the patency of the duct during the procedure. The lactiferous duct, lactiferous sinus, and the segmental duct and its branches were visualized in succession. The presence and appearance of any papillary lesions were noted, and the transductal distances from the nipple orifice to both the proximal and distal borders of the lesions were measured and recorded. The presence of atypical papillary lesions were specifically identified by the presence of bleeding, circumferential obstruction, or gross fungating projections.

Endoscopic biopsy

Endoscopic biopsy was performed when the presence of atypical papillary lesions was noted and when the biopsy was technically feasible. The fiberscope, which was covered with an outer cylinder, was inserted into the location of the suspected lesion through the ostium of the duct. Under the guidance of FDS, the outer cylinder was brought just up to the lesion. After the fiberscope was removed, a very thin inserted syringe was used to aspirate the lesion through the outer cylinder. With this technique, the fine-needle aspiration produced tissue fragments having the appearance of endoscopic biopsy specimens. These specimens were placed in 10% formalin for cytopathological examination.

Ductal lavage and cytology

The fiberscope was retracted, and the instilled saline was retrieved and processed for cytology. A double-lumen ductoscope was inserted in selected cases where retrieval of the instilled saline was limited, and ductal washings were obtained by irrigation and processed for cytology. Cytological analysis consisted of standard cytospin preparations and Pap and Diff-Quick staining. The cytological findings were grouped into the four following categories: clumps of ductal cells (>50 cells), clumps with mild atypia, clumps with severe atypia (cytologically malignant cells), and single ductal cells or small clumps. The number of ductal epithelial cells obtained on ductal lavage were compared to the number spontaneously present in the nipple discharge. For the purposes of this study, the four cytological categories were reduced to two—cytologically malignant and cytologically benign.

Pathological analysis

Select patients were subsequently subjected to breast surgery. Detailed histopathological analysis of the extirpated tissues was carried out to evaluate the intraductal abnormalities present.

Statistical analysis

Standard tests of significance were carried out. These included comparisons of differences among variables with the two-tailed Student's *t*-test and the Spearman's rank-based correlation to assess the relationship between the variables. The log-rank test was used to assess the univariate effect of certain variables on the presence or absence of an atypical vs typical intraductal lesion. Cox's proportional hazards model was used to assess these effects after adjustment for other covariates.

Results

Overview of findings

In this cohort of patients, ductoscopy was successful in visualizing an intraductal lesion in 166 patients (40%). In the majority of these patients, FDS examination detected lesions having the appearance of typical papillomas. However, in 10 patients, the intraductal lesion exhibited one of several atypical features, including bleeding, circumferential obstruction, and gross fungating projections (Fig. 1). In eight of these patients, the subsequent histopathology turned out to be DCIS (Table 1). In four of these eight patients, cytologically malignant cells were present either on endoscopic biopsy or ductal lavage (Fig. 2). In three additional patients, although FDS examination revealed a typical papilloma that was not biopsied, ductal lavage (washings) revealed cytologically malignant cells. All 11 patients were subsequently shown to have DCIS on surgical pathology review of the extirpated lesions.

Clinical characteristics of the breast cancer patients with nipple discharge

Eleven of 415 patients with nipple discharge (2.7%) were therefore found to have DCIS. The mean age of all 11 DCIS patients was 43 years old (range, 27–56). The average duration of nipple discharge was 3 months and the longest duration was 8 months. The clinical characteristics of the patients are summarized in Table 2: The most prominent symptom of the patients in this study was spontaneous unilateral bloody nipple discharge from a single duct.

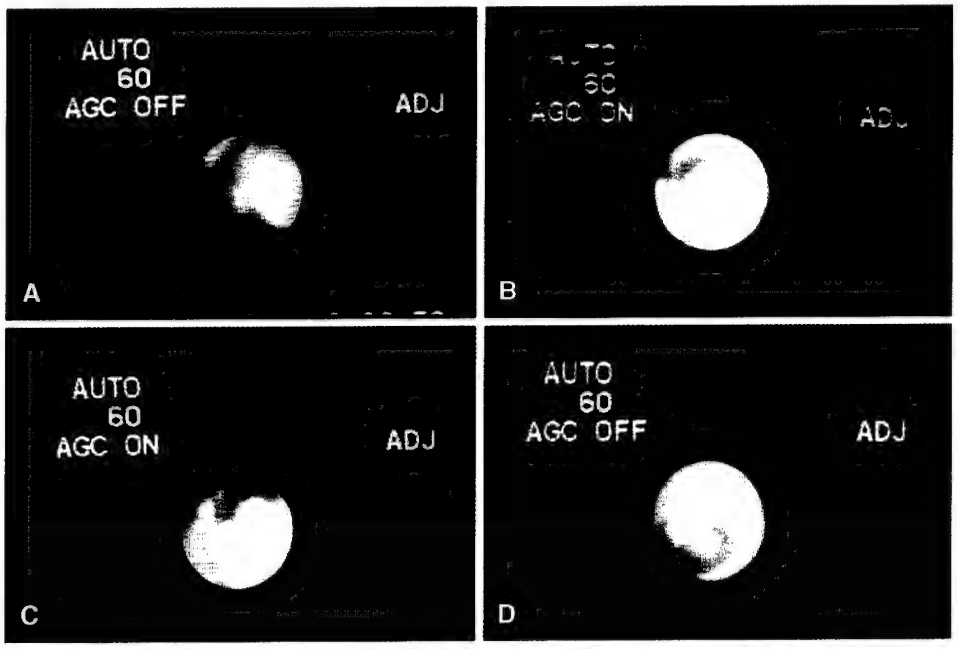

Fig. 1. Various appearances of the atypical intraductal papillary lesion suggesting intraductal carcinoma (DCIS): **a** a bleeding lesion, **b** a circumferential obstructive lesion with a roughened surface resembling a snow slide on a mountain, **c** an irregular fungating mass, **d** In contrast to these appearances, the typical intraductal papilloma, exhibited a narrow base and a smooth border and appeared as a poylp.

Table 1. + (suspicious) vs – (nonsuspicious) fiberoptic ductoscopy (FDS)[a,b] in the detection of intraductal carcinoma (DCIS) in women with nipple discharge

Ductoscopy results	+ histopathology (no. of patients)	–histopathology (no. of patients)
+ FDS (n = 10)	8	2
– FDS (n = 156)	3	153

[a] + FDS examination refers to the finding of an atypical papillary lesion; – FDS examination refers to the finding of a typical papillary lesion. All patients had a positive FDS examination from the standpoint of an intraductal lesion.
[b] Specificity, 99%; sensitivity, 73%; positive predictive value, 80%; negative predictive value, 98%

FDS findings

The intraductal lesions observed in the cases of DCIS tended to lie more distally than their benign papilloma counterparts observed both in this study and in a previous study [8]. The geographic distribution of the eight DCIS lesions detected by FDS were mainly in the first and second branches of the ductal system (Table 3) as opposed to the segmental and first branch, as was the case with intraductal papillomas. Predictably, the transductal distances from the nipple orifice to the lesion were greater for DCIS than for intraductal papillomas. The shortest transductal distance from the suspected lesion to the nipple orifice was 1.5 cm, the longest was 5 cm, and the average was 3.3 cm. In contrast, for intraductal papillomas, the shortest distance was 0.5 cm and the average was 2.7 cm.

The appearance of the eight DCIS lesions was quite different from the typical intraductal papilloma. The DCIS lesions exhibited bleeding (Fig. 1a), circumferential ductal obstruction (Fig. 1b), and irregular fungating masses projecting from the lumen (Fig. 1c). In contrast, the appearance of the intraductal papilloma was polyp-like, with smooth borders and a narrow stalk (Fig. 1d).

Fig. 2. An endoscopic biopsy specimen of intraductal carcinoma (DCIS) obtained through the ductoscope showing a clump of cytologically malignant cells.

Endoscopic biopsy findings

In two of the eight patients whose FDS examination revealed an atypical intraductal lesion, we were able to obtain a satisfactory endoscopic biopsy, which revealed tissue fragments of cytologically malignant cells (Fig. 2).

Ductal lavage cytology findings

Ductal lavage following ductoscopy in all cases that were studied dramatically increased the yield of cytologically interpretable ductal epithelial cells; it was 100-fold greater than the yield obtained with discharge fluid alone. The number of cells obtained averaged 5000 cells per washed duct, as compared to 50 in discharge fluid alone. The positive predictive value of FDS alone in detecting DCIS was 80%

Table 2. Clinical characteristics of the patients with intraductal carcinoma (DCIS) identified by fiberoptic ductoscopy (FDS) and/or ductal lavage cytology

Clinical characteristics	No. of patients	Percentage (%)
Menopausal status	11	100
Premenopausal	6	54.5
Postmenopausal	5	45.5
History	11	100
Family history of nipple discharge	1	9.1
Family history of breast cancer	3	27.3
Negative history	7	63.6
Localization of discharge	11	100
Unilateral	11	100
Bilateral	0	0
No. of discharge ducts	11	100
Single	10	100
Multiple	0	0
Duration of discharge	11	100
≤1 mo	5	45.4
2–3 mo	3	27.3
4–8 mo	3	27.3
Discharge characteristic	11	100
Bloody[a]	6	54.6
Serosanguineous	4	36.3
Serous	1	9.1
Mass—physical examination	11	100
No	8	72.7
Yes	3	27.3
Mammography	11	100
Microcalcifications	2	18.2
Architectural distortion (mass)	3	27.3
Negative	6	54.5

[a] Significant by univariate analysis, $p = .01$; insignificant in multivariate analysis, $p = .20$ when compared to FDS with respect to histopathology

Table 3. Anatomical distribution of lesions in patients with intraductal carcinoma (DCIS) identified by fiberoptic ductoscopy (FDS)

Location	No. of lesions	Percentage (%)
Segmental	1	12.5
First branch	3	37.5
Second branch	3	37.5
Third branch	1	12.5
Fourth branch	0	0
Total	8	100.0

(Table 1), but when combined with ductal lavage cytology, the positive predictive value increased to 100% (Table 4).

Comparison of FDS and ductal lavage with physical examination and mammography

Of the 11 cases of DCIS that were initially detected with a combination of FDS and ductal lavage cytology, six cases were completely negative on mammogram and physical exam (Table 2).

Discussion

FDS is gaining in popularity as thinner, longer, and more flexible fiberoptic scopes become available to permit greater visualization of the ductal system of the breast. Breast ductoscopy has evolved slowly over the past decade. In 1988, using a rigid endoscope with a 1.7-mm outer diameter [9], Teboul was the first investigator to succeed in observing the duct cavity under the guidance of ultrasound. In 1991, Makita et al. [4] established the use of blind intraductal biopsy of the breast for the pathologic diagnosis of nipple discharge through a rigid endoscope with a 1.25-mm outer diameter. The endoscope that this group used was similar to an arthroendoscope. Later, Okazaki et al. [6], in cooperation with the Fujikura Company, developed the breast fiberoptic ductoscopy system. This system made it possible to visualize the duct cavities and duct walls directly and project them onto a screen. This technique allowed for the visualization of even minute intraductal lesions.

Using this technique [8], we were able to effectively screen women with nipple discharge. In our initial study, we applied ductoscopy to 259 women with nipple discharge and analyzed the visual findings, the cytological washings, and the subsequent histopathology. In 92 of these women (36%), fiberoptic ductoscopy was successful in visualizing an intraductal papillary lesion. Of these visualized cases, 68 (74%) had a single papilloma, 21 (23%) had multiple discrete papillomas, and three (3%) had diffuse intraductal thickening that corresponded to diffuse papillomatosis on histopathological analysis. The overall positive predictive value of FDS screening was 83%. Ductal washings done at the time of ductoscopy were effective at obtaining representative exfoliated ductal cells that could be evaluated for the presence of clumps (>50 cells) or single ductal cells. The presence of clumps with positive FDS increased the positive predictive value to 86%. We concluded that fiberoptic ductoscopy offered a safe alternative to ductography in guiding subsequent breast surgery in the treatment of nipple discharge.

Because nipple discharge can occasionally herald the onset of intraductal carcinoma of the breast (DCIS), we decided to determine whether FDS and postductoscopy lavage might prove useful in determining which patient's nipple discharge was on the basis of DCIS. With this approach, 11 of 415 cases were shown to have DCIS prior to surgical extirpation of the lesion.

Bauer et al. reported that when DCIS is associated with nipple discharge, nipple discharge becomes a clinical marker for locally extensive disease [1]. When the discharge is due to DCIS, extensive intraductal spread of the disease may preclude breast conservation. Our results concerning the locations of the lesions and their extent of ductal involvement were not significantly different from what we previously observed for intraductal papillomas [8]. Although DCIS is located slightly more distally from the nipple orifice, it does not involve more than one ductal system and its extent of ductal involvement can be defined with FDS. With the help of FDS, we can determine the location and extent of the DCIS before surgery; this information can be of tremendous assistance in planning breast conservation surgery.

Some investigators have implied that ductography is the best method for preoperative evaluation of the nature and site of the lesion causing nipple discharge, but the accuracy rates reported for the detection of DCIS in the setting of nipple discharge in two recent studies were only 39% and

Table 4. Value of cytology in + (suspicious) vs − (nonsuspicious) fiberoptic ductoscopy (FDS) examination[a]

	Overall (n = 166)		+ FDS (n = 10)		− FDS (n = 156)	
Cytology	+ Path	− Path	+ Path	− Path	+ Path	− Path
Malignant cells (no. of patients)	7	0	4	0	3	0
Benign cells (no. of patients)	4	155	4	2	0	153
Specificity	100%		100%		100%	
Sensitivity	64%		50%		100%	
Positive predictive value	100%		100%[b]		100%	
Negative predictive value	97%		33%		100%[b]	

[a] Path, pathology + FDS examination refers to the finding of an atypical papillary lesion; − FDS examination refers to the finding of a typical papillary lesion. All patients had a positive FDS examination from the standpoint of an intraductal lesion

[b] Value greater than FDS alone (see Table 1)

70%, respectively [1,2]. The main reason for the low rates was that ductography could not discriminate between DCIS and papilloma. In our study, we show that FDS can discriminate effectively between DCIS and intraductal papilloma. Although we did not specifically compare ductoscopy with ductography in the management of patients with nipple discharge, it seems obvious that ductoscopy provides advantages over ductography in the routine management and workup of patients with nipple discharge(Table 5).

Ductoscopy allows direct visualization of the lesion and not just indirect shadowing. In comparison to ductography, ductoscopy allows for more precise localization and mapping of the lesion within the duct. This mapping is not dependent on the presence of obstruction. Ductoscopy, unlike ductography, permits intraductal sampling of the ductal cells through endoscopic biopsy and increases exfoliation of the cells in ductal lavage following ductoscopy 100-fold over that which occurs spontaneously in nipple discharge. In contrast, ductography obscures cytological detail and renders cytological evaluation impossible.

One of our important observations was that >50% of the patients with nipple discharge who had a DCIS lesion visible on FDS and/or detectable by ductal lavage had a negative physical examination and a negative mammogram (Table 2). In a recent study of women presenting with nipple discharge [1], mammography revealed subareolar microcalcifications in 28% and a tissue density in 14%, but it was completely negative in 52% of cases. It is especially in these latter cases that alternative detection methods such as FDS and ductal lavage would find utility. This study dealt with women who present with nipple discharge and the DCIS they harbor. But FDS and ductal lavage may hold greater promise as screening techniques for all women at high risk for breast cancer who are persistently negative on mammogram and physical exam.

In our previous study [8], we demonstrated that in 36% of women with nipple discharge, fiberoptic ductoscopy was successful in detecting an intraductal papillary lesion, with an overall positive predictive value of 83%. Therefore, from the standpoint of numbers of patients, the technique is cost-effective. Most of these intraductal lesions were intraductal papillomas. Only a minority were intraductal carcinomas. But we are advocating the technique for all women with nipple discharge, not just those whose discharge is due to intraductal carcinomas.

Table 5. Comparison of breast ductoscopy with breast ductography

Feature	Ductoscopy	Ductography
Proximal limits of lesion	Yes	Yes
Span of the lesion	Yes	No
Distal limits of lesion	Yes	No
Duct obstruction required	No	Yes
Direct visualization	Yes	No
Precise distances	Yes	No
Endoscopic biopsy	Yes	Yes
Cytologic analysis	Yes	No

The fiberoptic ductoscope and related equipment cost US $40,000. This amount represents a one-time cost for the silica fiberscope, the light source, the image monitor, and the image recorder. Each fiberscope can be reused for 100 patients. The cost of a fiberscope replacement is US $1000. Considering that patients with nipple discharge whose ductoscopy examination visualized a lesion were spared the cost of ductography and that patients whose ductoscopy/lavage were negative were spared the cost of surgical excision, the technique is quite cost-effective.

We cannot yet address the consequenses of diagnosing these lesions that are a cause of nipple discharge at a slightly later time when ductoscopy/lavage is not performed but replaced by more frequent screening mammograms or ultrasound examinations. However, considering that DCIS becomes a breast-threatening disease as it grows, and considering that the risk of invasion increases with size and duration, it seems intuitive that earlier diagnosis of DCIS is desirable.

The two techniques of ductoscopy and ductal lavage are intimately associated and should not be separated. Both ductoscopy and ductal lavage are done easily, and ductoscopy facilitates ductal lavage because it dilates the nipple orifices further. Ductoscopy both visualizes the lesion and determines its location and extent—determinations that cannot be made with ductal lavage alone. The cost of doing both techniques at the same time is no greater than doing either technique alone. Since we do not know in advance what the findings will be, it is not possible, nor would it be

desirable, to stratify the patients into receiving only ductoscopy or ductal lavage.

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Myoepithelial mRNA expression profiling reveals a common tumor-suppressor phenotype

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Abstract

A series of myoepithelial cell lines and xenografts derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast, and lung) exhibit common mRNA expression profiles indicative of a tumor-suppressor phenotype. Previously established myoepithelial cell lines and xenografts (HMS-#; HMS-#X) were compared to nonmyoepithelial breast carcinoma cells (MDA-MB-231 and MDA-MB-468, and inflammatory breast carcinoma samples, IBCr, and IBCw), a normal mammary epithelial cell line (HMEC) and individual cases of human breast cancer (zcBT#T), and matched normal human breast tissues (zcBT#N) (overall samples = 22). The global gene expression profile (22,000 genes) of these individual samples was examined using Affymetrix Microarray Gene Chips and subsequently analyzed with both Affymetrix and DChip algorithms. The myoepithelial cell lines/xenografts were distinct and very different from the nonmyoepithelial breast carcinoma cells and the normal breast and breast tumor biopsies. Two hundred and seven specifically selected genes represented a subset of genes that distinguished ($P < 0.05$) all the myoepithelial cell lines/xenografts from all the other samples and which themselves exhibited hierarchical clustering. Further analysis of these genes revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors, and proteinase inhibitors and decreased expression belonging to the classes of angiogenic factors and proteinases. Developmental genes were also differentially expressed (either over or underexpressed). These studies confirm our previous impression that human myoepithelial cells express a distinct tumor-suppressor phenotype.

Keywords: Myoepithelial cells; Tumor suppression; Expression profile; Microarrays; Hierarchical clustering; Angiogenic inhibitors; Angiogenic factors; Proteinase inhibitors; Proteinases; Developmental genes

Introduction

It has become clear that cancer cells come under the influence of important paracrine regulation from the host microenvironment (Cavenee, 1993). Such host regulation may be as great a determinant of tumor cell behavior in vivo as the specific oncogenic or tumor-suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves (Liotta et al., 1991; Safarians et al., 1996). Both positive (fibroblast, myofibroblast, and endothelial cell) and negative (tumor-infiltrating lymphocyte and cytotoxic macrophage) cellular regulators exist which profoundly affect tumor cell behavior in vivo (Cornil et al., 1991; Folkman and Klagsbrun, 1987). One host cell, however, the myoepithelial cell, has escaped the paracrine onlooker's attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, is thought to contribute largely to both the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and differentiating epithelial cells in health and to abnormally proliferating epithelial cells in precancerous disease states. Myoepithelial cells also form a natural border separating epithelial cells from stromal angiogenesis. These anatomical relationships suggest that myoepithelial cells may exert important paracrine suppressive effects on epithelium and endothelium and may inhibit the progression of ductal carcinoma in situ (DCIS) to invasive breast cancer and carcinoma-induced

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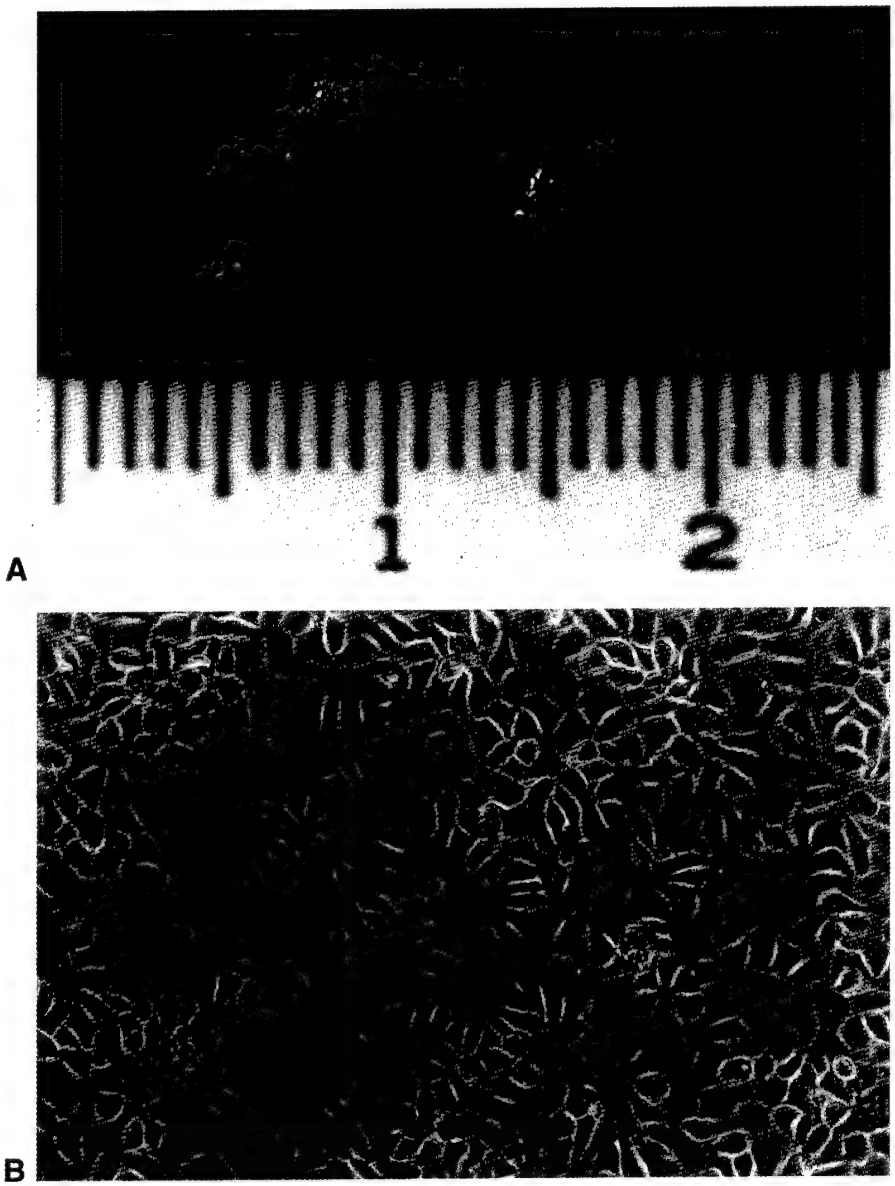
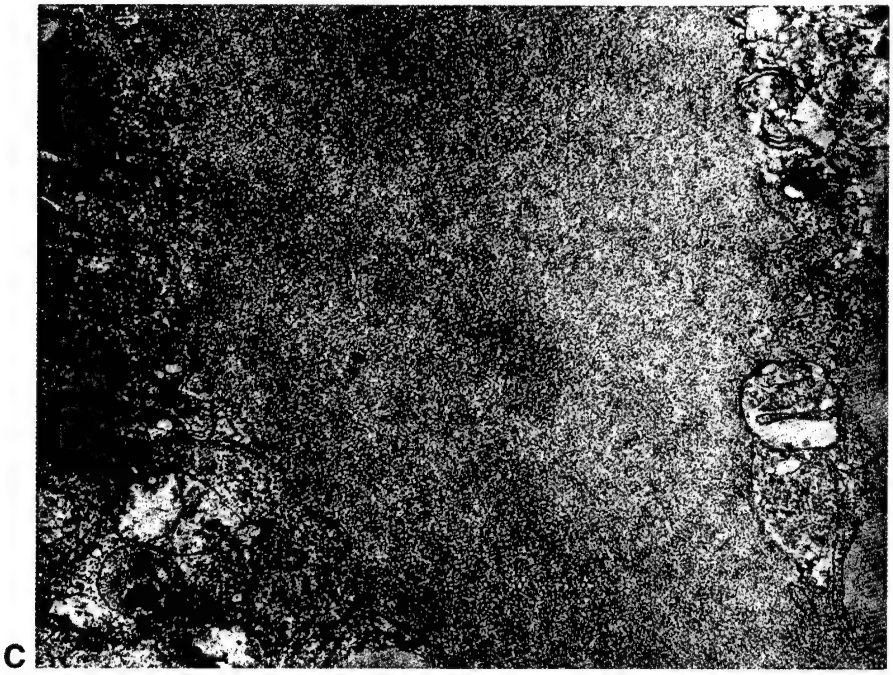


Fig. 1. (A) A typical myoepithelial xenograft, HMS-X, exhibits a white cartilaginous appearance due to the abundance of extracellular matrix; (B) a typical myoepithelial cell line, HMS-1, exhibits a polygonal cellular shape forming an epithelial-like monolayer; (C) ultrastructural studies of the xenograft, HMS-X, reveal a finely granular proteoglycan-containing extracellular matrix with absent fibroblasts, endothelial cells, and inflammatory cells; (D) karyotype of HMS-1 reveals a near normal diploid karyotype; (E) one gene product, maspin, was present immunocytochemically within the transformed myoepithelial cells of a typical myoepithelial xenograft, HMS-4X, and the same gene product was present within normal breast myoepithelial cells in situ (F). (A) Gross photograph; (B) phase contrast, ×200; (C) uranyl acetate, lead citrate, ×24,000; (D) karyotype with trypsin-Giemsa banding; (E) anti-maspin, immunoperoxidase, ×400; (F) anti-maspin, immunoperoxidase, ×400

angiogenesis. Additional evidence suggests that myoepithelial cells may also exhibit a natural autocrine suppressive phenotype. Myoepithelial cells rarely transform and when they do generally give rise to benign neoplasms and not malignant ones. There has been a paucity of studies on myoepithelial cells because they have been relatively difficult to culture and because tumors that arise from these cells are rare.

In previous studies we have successfully established immortalized cell lines and transplantable xenografts from benign or low-grade human myoepitheliomas of the salivary gland, breast, and lung (Barsky et al., 1988; Sternlicht et al., 1996, 1997) . We collected these tumors in the 1980/s under IRB and NIH Human Subject Protection Committee Review Exemption 4 which applied to all excess human tissues removed for either diagnostic or therapeutic purposes which would otherwise be discarded. We also collected a number of nonmyoepithelial breast carcinomatous tissues including inflammatory breast cancers (IBCr and IBCw) during this same time period. Under Exemption 4, the identities of the human subjects were kept anonymous. The myoepithelial lines/xenografts collected were designated HMS-#; HMS-#X, respectively. These abbreviations stood for human ma-



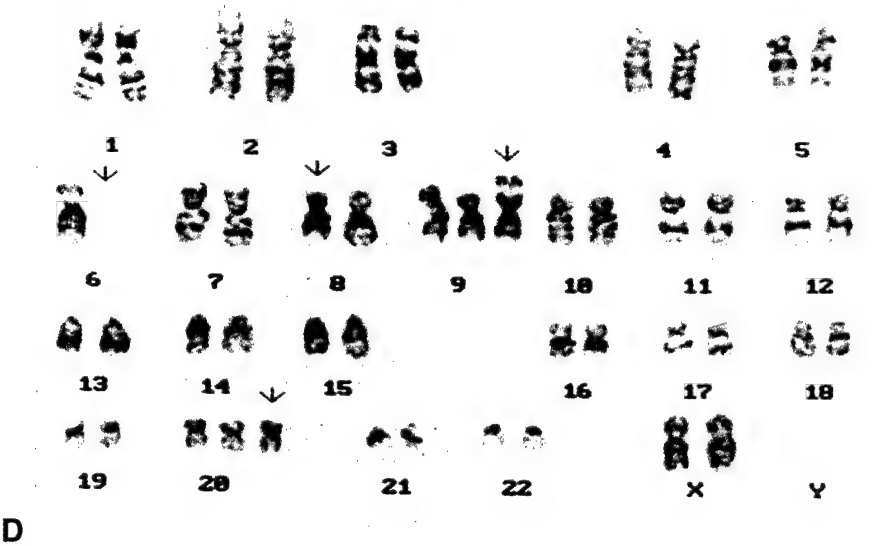


Fig. 1 (*continued*)

trix secreting line and xenograft, respectively, and referred to the chronological order of establishment. To date 4 lines/xenografts numbered HMS-1–6 and HMS-X–6X have been immortalized. HMS-2 and 2X and HMS-5 and 5X proved nonviable. The 4 established cell lines/xenografts displayed an essentially normal diploid karyotype and expressed identical myoepithelial markers as normal myoepithelial cells in situ. Unlike the vast majority of human tumor cell lines and xenografts which exhibited matrix-degrading properties these myoepithelial lines/xenografts like their myoepithelial counterparts in situ retained the ability to secrete and accumulate an abundant extracellular matrix. When grown as xenografts, the tumors were essentially avascular and their extracellular matrix was human and not murine in origin (Nguyen et al., 2000). When grown as a monolayer, one prototype myoepithelial cell line, HMS-1, exerted profound and specific effects on normal epithelial and primary carcinoma morphogenesis (Sternlicht et al., 1996). These studies supported our view that our established myoepithelial cell lines/xenografts could serve as primary myoepithelial cell surrogates. Because of our previous studies which suggested that human myoepithelial cells naturally exert a tumor-suppressive phenotype (Nguyen et al., 2000; Sternlicht et al., 1997), we decided to subject our human myoepithelial cell lines/xenografts to comparative mRNA expression profiling (gene chip analysis) to see whether the different myo-

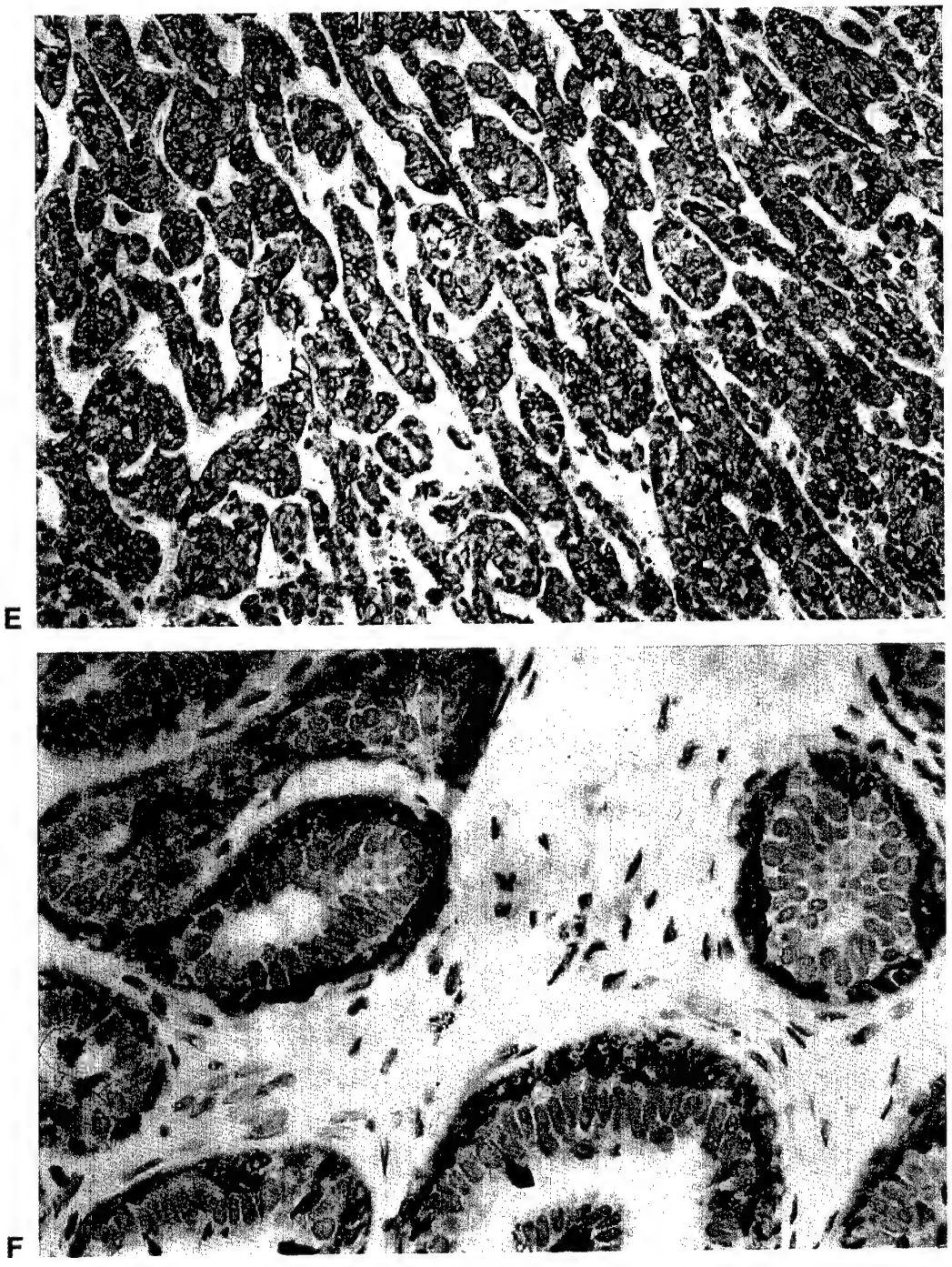


Fig. 1 (*continued*)

epithelial cell lines/xenografts group together and what gene clusters emerge.

Materials and methods

Source materials

Previously established myoepithelial cell lines and xenografts (HMS-1; HMS-X–HMS-6X) were subjected to standard gross, microscopic, ultrastructural, immunocytochemical, karyotype, and phase-contrast studies. These myoepithelial cell lines/xenografts were also compared to nonmyoepithelial breast carcinoma cells (MDA-MB-231, MDA-MB-468, IBCr, IBCw), a normal mammary epithelial cell line (HMEC) and individual cases of human breast cancer (zcBT#T), and matched normal human breast tissues (zcBT#N) (overall samples = 22).

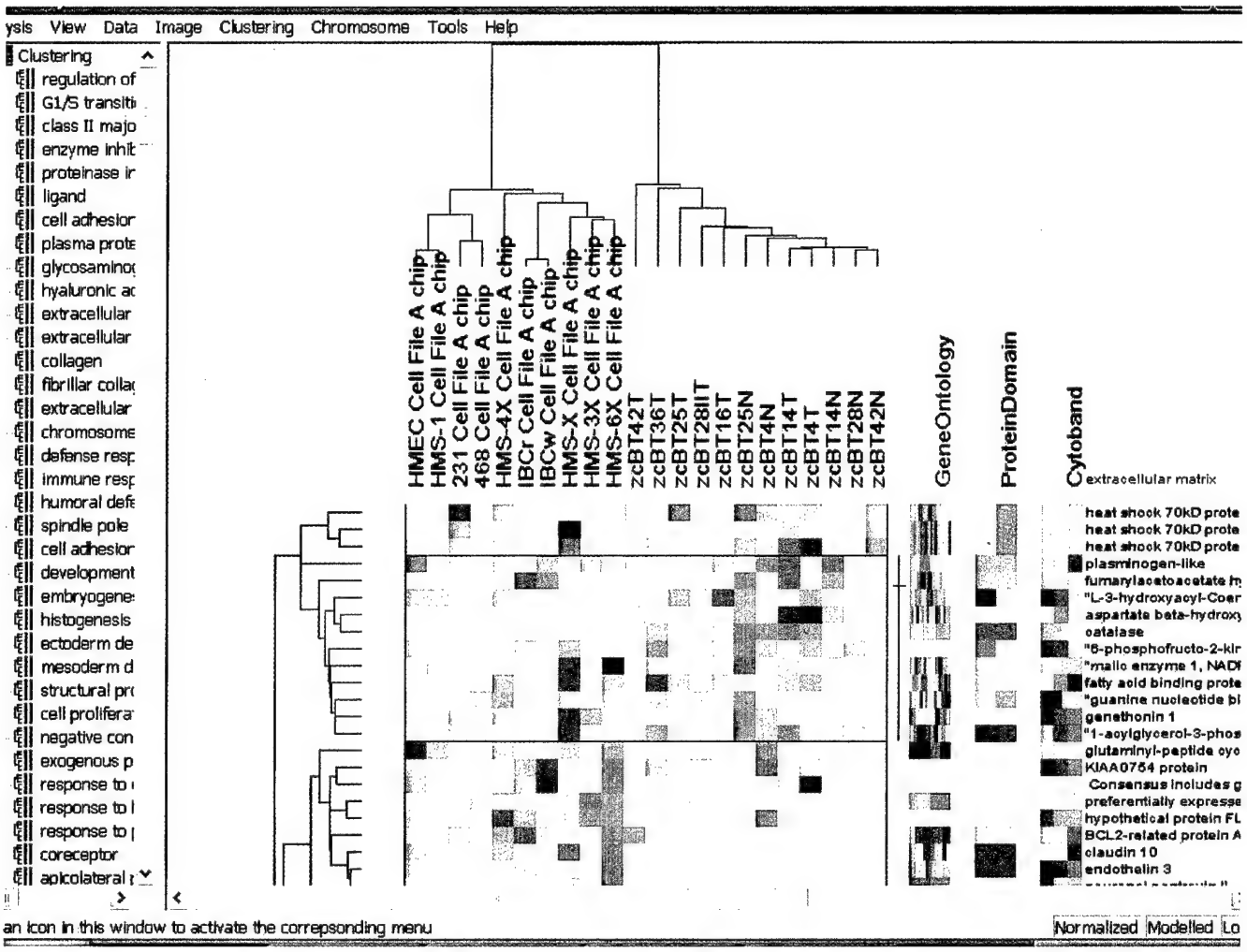


Fig. 2. Hierarchical clustering of 22 samples ("experiments") and 3200 genes that varied across all the experiments. Red indicates relative overexpression; blue, relative underexpression. The general pattern is that the myoepithelial samples are distinct from the nonmyoepithelial samples. Dendrogram, 3200 genes.

Hybridization and scanning

Ten micrograms of total RNA extracted from each sample was amplified by standard in vitro transcription methods and chromogen-labeled. Hybridization was carried out according to Affymetrix protocols and chips were scanned and gene intensities were recorded. All data were log 2-transformed with negative values set to 1. Ratios of gene expression were calculated by subtracting log 2 values.

Gene chip analysis

The expression levels were calculated by using DChip algorithms where individual sample expression was compared to the mean of all samples. Red indicated higher expression and green (or blue) indicated lower expression. Black indicated an expression level equal to the mean. Each gene chip analysis was termed an "experiment." The global gene expression profiles (22,000 genes) of these individual experiments were examined using Affymetrix Microarray Gene Chips and subsequently analyzed with both Affymetrix and DChip algorithms. An initial subset of 3200 genes that maximally varied among the experiments was selected. All the samples and all these genes were then subjected to hierarchical clustering. Based on the results of this initial analysis, a further subset of 207 genes representing genes which specifically distinguished ($P < 0.05$) the myoepithelial cell lines/xenografts from all the other samples and which themselves exhibited hierarchical clustering was used. The names and identities of the genes were recorded. Additionally filtered gene files, the subset of genes remaining after noninstructed forms of filtering, selected for genes that were varying across all the samples maximally. Genes with a 3-fold differential (either over or underexpressed) between the myoepithelial cell lines/xenografts and the nonmyoepithelial samples were rank-ordered. The classes of genes showing the highest fold differentials were recorded.

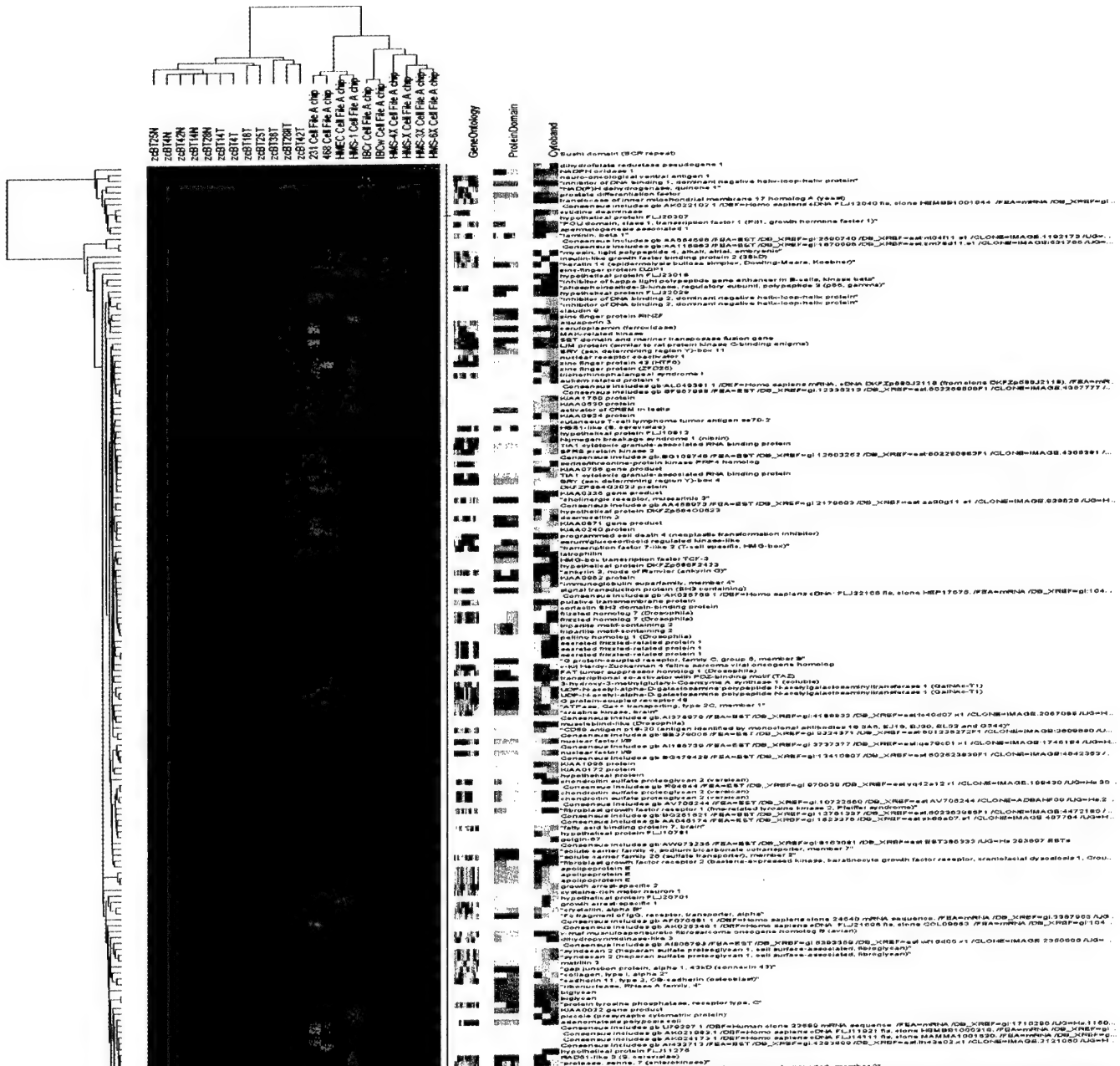


Fig. 3. Hierarchical clustering of 22 samples and 207 genes. Many of these genes were developmental genes, which represent the subset of genes which distinguish the myoepithelial samples from the nonmyoepithelial samples at a significance level of $P < 0.05$. The point of this dendrogram is to appreciate the checkerboard pattern of gene expression noted in the upper right corner of the dendrogram, which contributes to the hierarchical clustering of the myoepithelial samples into a distinct group:

Nonmyoepithelial	Myoepithelial
Red (overexpressed) green (underexpressed)	Green (underexpressed) Red (overexpressed)

Dendrogram, 3200 genes.

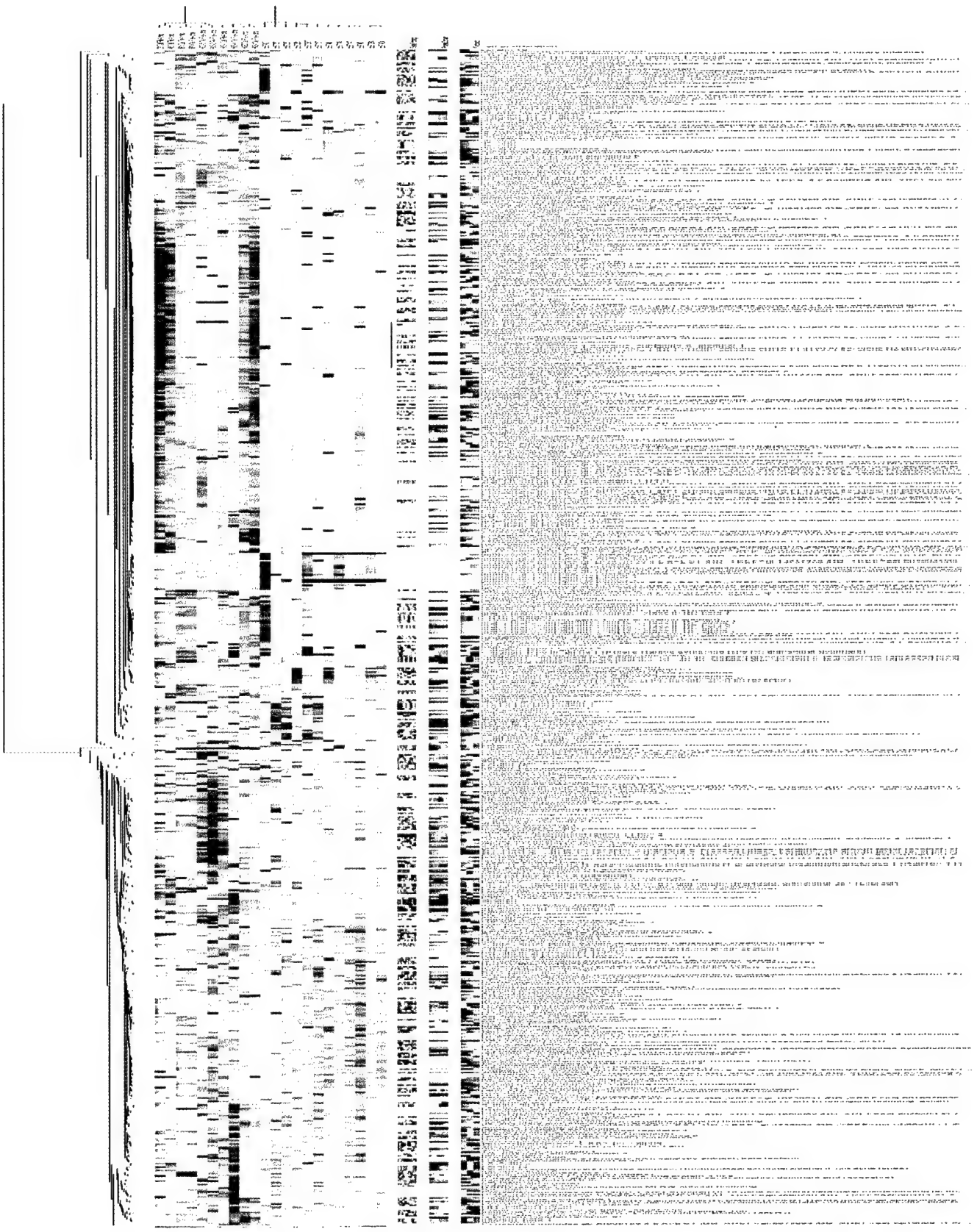


Fig. 4. Filtered genes are depicted which exhibit a 3-fold differential expression between the myoepithelial vs. nonmyoepithelial samples. In the upper left part of the dendrogram, myoepithelial underexpressed genes are depicted (blue); in the lower left part, myoepithelial overexpressed genes are depicted (red). The point of this dendrogram is not to depict the individual genes but rather the overall pattern of differential expression. Dendrogram, differentially expressed genes.

Table 1
Genes differentially expressed in myoepithelial vs non-myoepithelial cells

Class	Gene	Gene	Expression
Extracellular matrix			ALL ↑
	COL1A1	Laminin B	
	COL4A1	Nidogen/Entactin	
	COL4A2	BM90	
	HSPG2	Bamin	
	FN1	BM40/Osteonectin	
	Laminin A		
Proteinase inhibitors			ALL ↑
	Maspin	α1-AT	
	TIMP-1	31-kDa inhibitor	
	PN-II	PAI-1	
Proteinases			ALL ↓
	72-kDa gelatinase	Stromelysin	
	92-kDa gelatinase	uPA	
Angiogenic inhibitors			ALL ↑
	Thrombospondin-1	TIMP-1	
	Soluble bFGF receptors	Plasminogen	
	Plasminogen	Prolactin	
Angiogenic factors			ALL ↓
	bFGF	VEGF	
	aFGF	Angiogenin	
	TGFα	Platelet-derived ECG	
	TGFβ	Placental growth factor	
	TNFα	HB-ECGF	
	HGF		
Developmental genes			BOTH ⇅
	See Fig. 3		

Abbreviations used: COL1A1, type I collagen α1 chain; COL4A1, type IV collagen α1 chain; COL4A2, type IV collagen α2 chain; HSPG2, perlecan; FN1, fibronectin; TIMP-1, tissue inhibitor of metalloproteinase-1; PN-II, protease nexin-II; α1-AT, α1- antitrypsin; PAI-1, plasminogen activator inhibitor-1; uPA, urokinase-type plasminogen activator; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; TFGα, transforming growth factor α; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; platelet-derived ECGF, platelet-derived endothelial cell growth factor; HB-ECGF, heparin-binding endothelial cell growth factor.

Confirmation by Northern blot, Western blot, and zymograms

For select genes the findings on chip analysis were confirmed by either Northern blot, Western blot, or zymogram analysis according to established procedures.

Results

The human myoepithelial cell lines/xenografts subjected to the present microarray analysis exhibited a unique gross (Fig. 1A) and characteristic phase-contrast appearance (Fig. 1B). Ultrastructural studies of the xenografts confirmed the presence of abundant extracellular matrix devoid of murine stromal cells, inflammatory cells, and endothelial cells (angiogenesis) (Fig. 1C). These findings suggested that microarray analysis would reflect only human gene expression. Karyotype analysis of the transformed myoepithelial cell lines revealed only minimal deviations from a normal karyotype (Fig. 1D) suggesting that they would serve as a suitable normal myoepithelial cell surrogate. Furthermore the same gene products could be detected within the transformed myoepithelial cells of the xenografts (Fig. 1E) as within normal myoepithelial cells in situ (Fig. 1F). These findings suggested that subsequent microarray analysis would reflect the normal myoepithelial cell phenotype.

These myoepithelial cell lines/xenografts which had been derived from benign human myoepithelial tumors of diverse sources (salivary gland, breast, and lung) exhibited a common mRNA expression profile which was indicative of a tumor-suppressor phenotype (Figs. 2–5; Table 1). With hierarchical clustering, a significantly distinct grouping of the myoepithelial cell/xenograft samples was observed based on an analysis of 3200 genes (Fig. 2). With a subsequent analysis based on a subset of 207 genes ($P < 0.05$) even tighter grouping of the myoepithelial samples was observed (Fig. 3). Within this subset of 207 genes was an excess of developmental genes, both over and underexpressed (Fig. 3; Table 1). Additionally filtered genes, hierarchically clustered and rank ordered, exhibited a 3-fold differential (either over or underexpressed) between the myoepithelial samples and the nonmyoepithelial samples (Fig. 4). Analysis of these clusters revealed increased expression in genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors, and proteinase inhib-

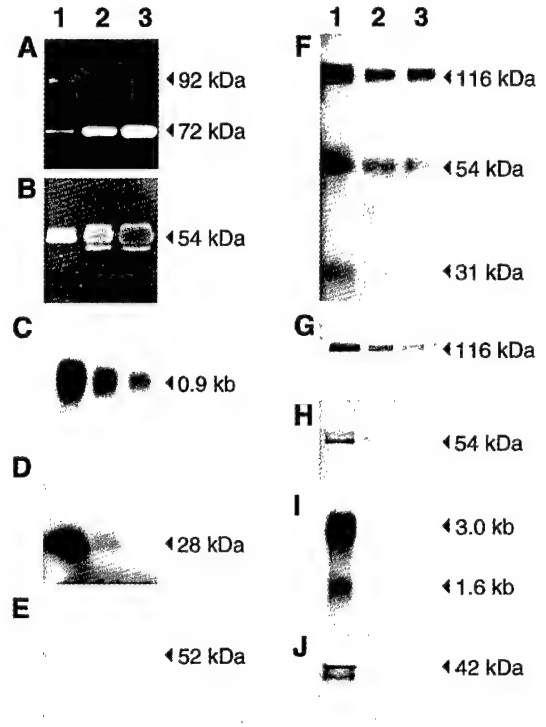


Fig. 5. Confirmatory pattern of expression of one prototype myoepithelial cell line, HMS-1 (lane 1), compared to two prototype nonmyoepithelial cell lines (lanes 2 and 3): direct gelatin zymography depicting the 92- and 72-kDa type IV collagenases confirms decreased expression in the myoepithelial cell line (A); direct fibrin zymography revealing the 54-kDa uPA also confirms decreased expression of this proteinase in the myoepithelial cell line (B); TIMP-1 expression reveals increased expression in the myoepithelial cell line by both Northern blot (C) and reverse zymography (D); another proteinase inhibitor, PAI-1, shows similarly increased expression in the myoepithelial cell line by reverse fibrin zymography as a 52-kDa lysis-resistant band (E); trypsin-like serine proteinase inhibitor expression is also increased in the myoepithelial cell line as demonstrated by reverse trypsin zymography as resistant bands of 116, 54, and 31 kDa (F); equivalently loaded Western blots using PN-II and α1-AT antibodies confirming increased expression in the myoepithelial cell line of two of the inhibitors: the 116-kDa PN-II (G) and the 54-kDa α1-AT inhibitor (H); the expression of maspin, a serine proteinase inhibitor and angiogenic inhibitor, is also dramatically increased in the myoepithelial cell line by Northern blot as 3.0- and 1.6-kb mRNA transcripts (I) and Western blot as a 42-kDa protein (J). See Table 1 for abbreviations used.

itors and decreased expression of genes belonging to the classes of angiogenic factors and proteinases (Table 1). Numerous expressed sequence tags also clustered within each of the aforementioned classes. Northern blot, Western blot, and functional assays, e.g., zymograms confirmed the increased/decreased expression of selected genes noted on microarray analysis (Fig. 5).

Discussion

It is the hope of high throughput approaches that the totality of gene expression or at least a subset of this totality will prove useful in hypothesis generation and eventual hypothesis testing. In the case of myoepithelial cells, the fact that diverse cell lines/xenografts derived from different benign neoplasms from disparate sites all cluster together supports the hypothesis that the myoepithelial phenotype is a distinct entity worthy of further study. When one considers the fact that myoepithelial cells rarely transform and when they do, transform to benignity and not malignancy (Guelstein et al., 1993), one could generate the following hypotheses: (1) that myoepithelial cells have an endogenous program of chemoprevention and (2) that myoepithelial cells have an endogenous program of metastasis suppression. Both of these programs would be contained in their pattern of global gene expression and perhaps in the subset of genes specifically characteristic of the myoepithelial phenotype (Zhang et al., 2000). One gene or the orchestrated expression of many genes may exert chemoprotective effects and similarly one gene or the orchestrated expression of many genes may suppress metastasis formation or contribute to tumor dormancy. Our microarray analysis employing hierarchical clustering demonstrated the strong grouping of the class of myoepithelial samples which clustered distinctly and very differently from nonmyoepithelial breast carcinoma cell lines, normal breast, and breast tumor biopsies. Hierarchical clustering of the genes involved revealed within the myoepithelial samples groups of genes belonging to the classes of extracellular matrix proteins, angiogenic inhibitors, and proteinase inhibitors, all of which showed increased expression, and groups of genes belonging to the classes of angiogenic factors and proteinases, all of which showed decreased expression. Numerous expressed sequence tags clustered with each of the aforementioned classes. When one considers the appearances of the myoepithelial xenografts, one is struck with their gross resemblance to cartilage. This appearance is due to the prominence of their extracellular matrix. One would obviously then anticipate that the class of genes which would show increased expression would be extracellular matrix genes and this was indeed the case. Myoepithelial tumors are prototype benign neoplasms which do not exhibit true invasion and which are devoid of any appreciable angiogenesis (Nguyen et al., 2000; Sternlicht et al., 1997). From these observations one would predict that they would exhibit increased expression of proteinase inhibitor and angiogenesis inhibitor genes and decreased expression of proteinases and angiogenic factor genes and this too was the case. One would not, however, predict that developmental genes would also be differentially expressed (either increased or decreased) in the myoepithelial samples. The hierarchical clustering of presently uncharacterized expressed sequence tags with each of the above-mentioned classes of genes suggests functional similarities and the possibility that they represent new angiogenic inhibitors or proteinase inhibitors which await discovery. When one considers that the myoepithelial phenotype is tumor suppressive and that myoepithelial cells have a built in chemoprevention and metastasis-suppression program, one could envision using this high

throughput gene expression profiling approach to identify candidate genes and candidate patchways which are either chemopreventive or metastasis suppressive in nature.

Acknowledgments

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bruin-on-line

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Sent: Friday, July 02, 2004 1:15 PM
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Dr Susan Love Research Foundation
office 310 230 1712
mobile 310 403 4486

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To: slove@earthlink.net
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Anatomy of the nipple and breast ducts revisited

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Summary

Background Increasing interest in the intraductal approach to the breast has necessitated revisiting the anatomy of the breast.

Methods Using six different complementary *in vivo* and *in vitro* approaches we determined the number, distribution and anatomical properties of the ductal systems of the breast which extend from the nipple orifices to the terminal duct lobular units.

Findings Over 90% of nipples studied contain 5-9 ductal orifices, generally arranged as a central group and a peripheral group. All orifices are normally occluded by keratin plugs which can be disrupted by lactation, external suction or retrograde wicking of injected lymphazurin dye, creating patent orifices. Each nipple orifice communicates with separate non-anastomosing ductal systems which extend to the terminal duct lobular units.

Interpretation Increased knowledge of the ductal anatomy of the breast and our ability to access the nipple ductal orifices will provide the foundation for the intraductal approach to the breast.

Introduction

Recent attempts to access the ductal systems by endoscopy[1,2] or lavage[3] have made it imperative that we obtain a more accurate description of the ductal anatomy than presently exists. Since the studies of Wellings,[4] we have known that breast cancer is primarily a disease of the terminal duct lobular units. Detailed histological descriptions of the terminal duct lobular units and electron micrographs of epithelial microvilli obscure the fact that we do not know how they connect to the nipple orifices. Astley Cooper in one of the earliest studies on record (in1845)[5] described over 200 dissections of breasts where he reported not only on the anatomy but also the development and involution of the breasts of women, men and assorted animal species. Not until 1972 was there any attempt to study the ductal anatomy of the breast systematically with modern techniques. In that attempt, Otto Sartorius[6] obtained over 2000 ductograms and initiated an analysis of the ductal anatomy. Teboul published his elegant atlas of ultrasound and ductal echography of the breast in 1995.[7] He described over 6000 ultrasound studies of the breast ducts and lobules subtitling his book, "The introduction of anatomic intelligence into breast imaging." More recently, Ohtake *et al*[8] did computer simulations based on surgical quadrantectomies to analyze ductal anatomy and Moffat and Going reported a three dimensional computer model based on sub gross

coronal slices of an autopsy breast.[9] These independent studies of the ductal anatomy were contradictory in the number of ductal orifices, the number of different ductal systems and the presence of anastomoses among different ductal systems. In an attempt to resolve these controversies and to facilitate new screening and chemopreventive approaches, we determined both the number, distribution and anatomical properties of the ductal systems by six different, though complementary, approaches all based on the successful identification of and access into the nipple ductal orifices.

Patients and methods

All studies were approved by the UCLA Human Subjects Protection Committee and Institutional Review Board.

Approach #1: Direct observations of the number and distribution of nipple ductal orifices by in vitro serial sectioning and three dimensional digital image analysis.

In order to better understand the milk duct orifices we performed an extensive evaluation of 10 nipples which had been fixed in formalin and paraffin, embedded and subjected to coronal sectioning. 15μm step sections were taken through each nipple (an average of 1000 sections per nipple). Sections were stained with hematoxylin and eosin and where appropriate combined with anti-cytokeratin immunocytochemistry to detect the ductal orifices and to distinguish them from sebaceous and sweat ducts and isolated keratin plugs. The two dimensional sections indicating the presence of ductal orifices were subjected to additional three dimensional digital image analysis which utilized a digital imaging system, composed of a Leitz Dialux microscope linked to a Vidicon camera, an IBM PC with PCVision digitizer, and Microscience software.

Approach #2: Direct in vivo observations of ductal orifices in lactating and non-lactating women.

To determine the number and pattern of ductal orifices, we decided to initially study lactating women. Two hundred and nineteen lactating women were enrolled in this study. This would insure that the orifices that we identified were indeed ductal orifices and not sebaceous glands or sweat ducts as originally described by Cooper and Sartorius.[5,6] With the assistance of La Leche League (a local and national breast feeding support group) and a local breast feeding support network located at the "Pump Station," a commercial source of breast pumps, we approached the lactating women. After obtaining informed consent, women were asked to empty their breasts in order to reduce pooling of milk on the nipple. A trained observer determined the number and location of milk-producing nipple ductal orifices before pooling occurred. This approach tended to underestimate the number of orifices since it was more difficult to count many orifices than a few. The same observer studied all of the women in the study in an effort to minimize inter-observer bias. Immediately after visualizing the lactation the observer diagrammed the orifices on a prepared grid. The location of the orifices was relationally characterized since the position of the women a nd their breasts were not perfectly standardized. Nonetheless, the resulting observations estimate the number of nipple duct orifices and their approximate location. We studied 219 lactating women. We performed a K-means cluster analysis[10] to determine empirically the number of nipple orifices and their pattern.

Approach #3: Direct observations of the number and distribution of nipple ductal orifices by in vitro and in vivo transareolar dye injection

In an attempt to confirm our in vivo and in vitro findings we studied 13 detached breasts and one in vivo. In mastectomy specimens (13 breasts) and 1 patient, a transareolar dye injection technique was employed to identify the ducts. This technique made use of the fact that the lactiferous sinuses taper as they course to the surface of the nipple, therefore causing a natural wicking of dye up the duct to the nipple. 1-2 cc of water-soluble lymphazurin was introduced transareolarly into the base of the nipple with a syringe. The dye diffused into lactiferous sinuses and wicked up via capillary action causing dark blue spots to appear on the nipple surface. The number and location of the orifices was recorded. This procedure was repeated in a woman using lymphazurin mixed with lidocaine and was tolerated without difficulty. The nipple ductal orifices were identified as in the *in vitro* studies.

Approach #4: Direct observations of the number, distribution and physical properties of the underlying ductal systems of the breast by in vitro ductal orifice cannulation, dye instillation and histological sectioning.

After identifying the nipple ductal orifices with the transareolar dye injection as described in Approach #3, the orifices were cannulated and

dilated with guide wires prior to the placement of a single or double lumen catheter. In each nipple ductal orifice so catheterized a water-insoluble dye of a different color was instilled and the breasts were sectioned one hour later. Serial horizontal sections under the nipple and throughout the breast were taken to demonstrate the ductal profiles and the colored dyes they contained.

Approach #5: Calculations of the number and location of ducts and nipple ductal orifices based on the retrospective analysis of the archival ductograms of Sartorius.

In an attempt to determine the number and pattern of the ductal systems within the breast, we then analyzed 1312 archival ductograms. These ductograms had been performed by the late Otto Sartorius, a breast surgeon in Santa Barbara, in the 1960's-70. These ductograms represented 656 distinct ducts (two cross sectional views of each duct) in 470 women. The ductograms had been classified under Dr Sartorius' tutelage into ten categories based on observation. We elected to reexamine the ductograms using a different method to confirm Sartorius' classifications.

The approximate location of the center of the duct on the chest wall and the volume of the duct was determined for each ductogram by first measuring the areas in the region of the duct to either side in the two dimensional photograph. A planimeter was used to outline the extent

of the duct in relation to the total area and the areas on each side. The nipple was not included in the measurements. The back wall of the breast was defined as the rib cage, muscle or edge of the ductogram. The back wall was chosen which would yield the smallest area while still including the entire duct. For example if the ductogram showed both the muscle and the rib cage the muscle would be the back wall if it enclosed the entire duct. If the duct extended past the muscle wall the rib cage would be used as the back wall. When the ductogram didn't include the muscle or rib cage (i.e., did not include the whole breast on the film), the total breast measurement was deemed inaccurate. In our analysis we assumed that the breast was a hemisphere, the cross sections were semicircles and the ductal shape was triangular. We divided the semicircular cross-section into three sections: B was the section of the ductogram; A was the section lateral to the ductogram and C was the section medial to the ductogram. Using A, B and C from each cross sectional view (measured quantities) we defined and calculated:

X duct = location of the center of the duct, side to side (medial lateral) view

Y duct = location of the center of the duct head to foot (inferior superior) view

L_1,L_2 = length of duct respective views

V = volume of duct = function of L_1,L_2

Using the measured quantities: A, B and C from each cross sectional view where the total area in that view of the breast is standardized to one, we defined:

xduct=A_{ML}+0.5 B_{ML} to be the center of the duct in the medial lateral view

yduct=A_{IS}+0.5B_{IS} to be the center of the duct in the inferior-superior view

B_{ML}*B_{IS}=the relative volume of the duct

We performed a K-means cluster analysis[10] to determine empirically the number of ductal systems and their characteristics. We clustered (grouped) the ductograms/individual ducts based on the calculated chest-wall location of their centers (xduct, yduct). The criteria of Hartigan[10] of a large relative reduction in within-cluster variance was used to arrive at the number of clusters. The FASTCLUS procedure in the SAS computer package was used to carry out the clustering computations.

Approach #6: Confirmation of the value of previous ductal anatomy studies in surgical patients with mammographic findings.

This approach involved selected ductal lavage of lymphazurin-identified nipple ductal orifices with mammographic correlation in 6 women (7 breasts) who were scheduled for surgery. This study was conducted in Chile and also approved by the National Cancer Institute of Chile. The women scheduled for surgery for mammographically detected lesions were entered in the study. After informed consent had been

obtained, the breast was prepped and draped in a standard sterile fashion. The nipple was anesthetized using 0.5% lidocaine. The mammogram was studied and the lesion was localized to a particular sector of the breast. The involved ductal system was predicted to connect to either the central or peripheral group of ductal orifices. These calculations were used to identify the likely site of the corresponding nipple duct orifice. The transareolar dye injection technique (Approach #3) was then employed to identify the nipple ductal orifice. The orifice was then cannulated (Approach #4) and a double lumen catheter was then inserted into the duct for a distance of less than 1 cm. One cc of 0.5% lidocaine was instilled into the duct to further anesthetize it and then 10 cc of saline was lavaged. The fluid was centrifuged and stored in Cytolyt (Cytyc, Inc., Boxborough, MA), analyzed cytologically and correlated with the final pathology.

Role of the funding source: The Department of Defense sponsored the work but had no role in the study design, in collection, analysis and interpretation of data, in writing and report, or in the decision to submit for publication.

Results

Approach #1: Direct observations of the number and distribution of nipple ductal orifices by in vitro serial sectioning and three dimensional digital image analysis.

Analysis of 10 nipples revealed that the surface contained numerous indentations which are the sites of keratin plugs. The vast majority of these keratin plugs (>90%) are not associated with anything in the dermis (Figure 2A). Slightly under 10% are associated with either sebaceous glands (Figure 2B) or sweat or apocrine ducts. In 1,000 serial sections (15μm each) of each of 10 nipples, 5-9 ductal orifices/nipple could be demonstrated (Figure 2C, 2D). In these areas very thin ducts could be seen coursing up through the dermis and emerging at a site of a keratin plug (Figure 2C). Anti-cytokeratin immunocytochemistry was very helpful at highlighting these ducts and distinguishing them from sebaceous glands (Figure 2D). The lumens of the ducts which coursed up through the nipple were much smaller (1/10 –1/100) of the size of the much larger lactiferous sinuses which lay deep to the nipple. There were no differences between the size and shapes of the keratin plugs which occluded the lumens of the nipple ductal orifices and those which lay over sebaceous glands, sweat ducts or stood alone. Three dimensional digital image analysis of the ductal locations was consistent with a central and peripheral pattern of ducts.

Approach #2: Direct in vivo observations of ductal orifices in lactating women.

In the lactating women, data was collected on 424 nipples in 219 women. The mean number of nipple duct orifices was 5 with an extreme range of 1-17. The patterns were symmetrical. 98.8% of women had 13 or fewer nipple ductal orifices. (Table 1) A two-way histogram shows the relative frequency/numbers of the nipple openings by location (Figure 1A). Darker shading indicates higher relative numbers in that region of the nipple. The most common location was in the center of the nipple. We performed a K-means cluster analysis[10] to determine empirically the number of nipple orifices and their pattern. The nipple openings grouped into 13 clusters. The mean location and the standard error of the mean location, on the coordinates defined as x and y of the nipple openings in each of the 13 empirical clusters is depicted (Figure 1B]. Almost every woman had a duct orifice in the center of her nipple (cluster 2) and a second one in the central area but oriented to the upper outer quadrant (cluster 11). The overall pattern could be described as variable but the most consistent finding was a group of "central" orifices: one central with the others just above or below the central fold [Figure 1C]. In addition, there are more peripheral openings with upper lateral, upper medial and lower lateral being most common. This pattern was consistent with the tear shape of the breast.

Approach #3: Direct observations of the number and distribution of nipple ductal orifices by in vitro and in vivo transareolar dye injection

In all 13 of the mastectomy specimens, the transareolar dye injection technique was effective at labeling some nipple ductal orifices. By random diffusion the dye entered the lactiferous sinuses and wicked up via capillary action causing dark blue spots to appear on the nipple surface over time and marking the spot of the nipple ductal orifices. The uninjected nipple gave a suggestion as to the location of the ductal orifices (Figure 3A). However, with this transareolar dye injection approach, the first of the first two ductal orifices could be identified within 10-20 seconds (Figure 3B). These were usually the central ducts. Later (within 2-3 minutes), 3-7 additional ductal orifices appeared (Figure 3C). These tended to be the more peripheral ducts. Eventually the entire nipple turned blue but the location of all of the ductal orifices could still be discerned (Figure 3D). In addition to coloring the ductal orifices the injection of dye caused the nipple to be turgid and revealed orifices which had previously been hidden in folds of the nipple. There was a learning curve to this technique. Ductal orifices were identified by this technique in the first 8 cases but they were not all able to be cannulated and confirmed. The final five cases demonstrated all of the ductal orifices which were then confirmed with cannulation and histologic evaluation (see approach 4, Table 2). The transareolar dye injection approach also indicated that there were between 6 and 8 ductal

orifices/nipple. In the single patient studied with this approach, 8 nipple duct orifices were identified without difficulty.

Approach #4: Direct observations of the number, distribution and physical properties of the underlying ductal systems of the breast by in vitro ductal orifice cannulation, dye instillation and histological sectioning.

This study was carried out in 13 detached breasts mentioned above. After initial difficulty in cannulating the ducts (cases 1-8) we were able to cannulate all of the ducts (7-8) and demonstrate histologically successful instillation of dye that reached the recesses of the duct lobular units (Figure 4A) in the last 5 cases (9-13). Instillation of different dyes in separate nipple duct orifices revealed different color profiles in different ductal systems which often co-existed in the same breast quadrant (Figure 4B). In no case of these multiple colored instillations was there admixing of colors, suggesting that even when different ductal systems are in close juxtaposition, there are no communications or anastomoses among them. These different ductal systems could easily be misinterpreted as being the same ductal system on routine hematoxylin and eosin staining.

Approach #5: Calculations of the number and location of ducts and nipple ductal orifices based on the retrospective analysis of the archival ductograms of Sartorius..

For those ductograms with the age of the patient known (n=400, range 14 to 88 years of age, median age 40 years), we analyzed the relationship between age and xduct, yduct, and V. Significant ($p<0.01$) negative correlations between age and yduct and between age and V, and a significant positive ($p<0.05$) correlation between age and xduct, were found. The significant negative correlation between age and V persisted when controlling for the effects of xduct and yduct upon V through a multiple linear regression analysis. Similar correlations were found when we repeated the analysis, choosing a single ductogram per woman (n=286 with age known) to avoid the technical problem of within-subject correlation. The results of the distribution of the ducts was depicted in a 2-D histogram (Figure 5A). This histogram showed the relative frequency/concentration of the ducts by ductal location (xduct, yduct). Darker shading indicates higher relative numbers of ducts projected in that region of the chest wall.

Cluster analysis of the ductograms was not tight. Using all 656 ductograms, there was no convergence of the clustering algorithm into fewer than 20 clusters. Using one ductogram per woman (selected at random), there were 11 clusters of the 474 ductograms. The mean location and its standard error of each of these clusters is depicted (Figure 5B). Using only ductograms from women of age 50 and under (304 ductograms for n=220 women), there were 13 clusters. The mean location and its standard error of each of these clusters is depicted

(Figure 5C]. The general pattern of ducts in our analysis of the Sartorius ductograms indicated a central duct directed towards the chest wall from the nipple. The approximate distribution of ducts is as follows: There is a central duct. This is surrounded by a central upper duct, a central medial duct and a central lateral duct. This grouping forms the central circle of ductal orifices. More peripherally are the upper lateral and upper medial ducts, the lower lateral and lower medial ducts and the deep lateral duct. The deep duct describes a pattern where a narrow duct travels towards the chest wall and then arborizes in the upper outer quadrant. Other interesting findings include the fact that the ductal systems become narrower and longer with age. This is undoubtedly a result of ptosis which increases with age and is reflected on the mammograms.

The results of our analysis of the Sartorius ductograms match our findings in Approach #1 - #3. Although these approaches used different cohorts of women and although they were not an exact match, they did corroborate certain findings: there are two groups of ducts -- a central collection of ducts and ductal orifices and a more peripheral group. This overall distribution is better described as concentric circles rather than a clock face. In visualizing these ducts it is important to remember that the breast is not two-dimensional. The ducts do not all extend in a radial fashion from the nipple; rather some travel back from the nipple toward the chest wall. One way to visualize the ducts is to picture each orifice as

projecting toward the chest wall. The area covered by that duct as depicted on the chest wall represents the area of that ductal system (Figure 6).

Approach #6: Confirmation of the value of previous ductal anatomy studies in surgical patients with mammographic findings.

Our final approach was to confirm the clinical utility of this analysis. Six women were studied with no untoward events. Seven breasts were catheterized. Of the seven ducts that were washed, four (57%) had confirmation by ductogram and mammography that the correct duct had been cannulated. In one there was no dye in the breast but some which spilled on to the patient. In another the wrong duct was cannulated and a second was attempted without success. In the final one the lesion could only be visualized on ultrasound. Cytology of the washes correlated completely with the final pathology, with 3 cases of benign ductal cells and 1 case of malignant cells. It should be noted that three of the four successful cannulations were in the last two women, hinting at a learning curve for this procedure.

Discussion

Our findings with our multiple approaches all indicate that > 90% of nipples contain 5-9 ductal orifices distributed in two groups: central and peripheral. The central ducts do not extend in a radial fashion from the nipple but rather travel back from the nipple toward the chest wall while the peripheral ducts drape over the central ones in a radial fashion. Separate ductal systems may lie in the same quadrant in close juxtaposition to each other but they do not connect or anastomose. Central ducts more readily discharge with lactation, yield nipple aspirate fluid (NAF) upon suction, label more quickly with transareolar dye injection and hence are more easily cannulated and lavaged.

While we have demonstrated in the present study that each nipple ductal orifice marks a separate ductal system, we have not answered the question whether some minor sequestrations exist; that is, whether some ductal lobular units do not communicate with a nipple ductal orifice. Obviously this is an important question for sampling of cells by ductal lavage since epithelial precancerous proliferations in sequestrations could not be sampled through this method. In the mastectomy specimens where all the nipple ductal orifices were cannulated and dye instilled, we could demonstrate luminal dye in all the ductal systems present in random sections. Still, minor sequestrations could not be excluded.

Our anatomical findings challenge conventional dogma. Most textbooks of anatomy state that there are between 15-20 ducts and imply that there are 15-20 nipple ductal orifices. As a result of our studies we feel that many of these orifices represent sebaceous gland openings and not true ductal orifices. Astley Cooper in 1845[5] injected wax into the milk ducts in an attempt to outline their anatomy he described only being able to inject at most 12 lactiferous ducts and more commonly 7-10. He noted, however, that there were also "tubercles" which were separate from the ducts. This finding of two different types of nipple orifices, true ductal orifices and sebaceous gland orifices, was further substantiated and expanded by Otto Sartorius describing his experience in over 1000 ductograms.[6] He describes two types of openings: those of true mammary ducts and what he termed secretory gland openings. He concluded that there are five to seven true mammary duct orifices and that the other openings were sebaceous glands. These glands vary in length from 1-4 cm, have no branches and do not connect to the ducts. Although it is not known whether these "non ductal tubular" structures represent sebaceous glands, Cooper's wax model matches Sartorius' ductogram almost exactly. More recently and in a smaller series, Dietz *et al*[11] agreed with Sartorius' observation of 5-9 ducts, stating that they were able to cannulate 2-5 ducts per specimen. They go on to describe a finding that was consistent with that described by Cooper and Sartorius.[5,6] Finally, Going has done an extensive 3-D reconstruction of

a nipple and found 27 "ductal" appearing structures; but about .8-1 mm from the nipple most of them disappear into a skin appendage leaving only 7 to continue to surface through the nipple as milk duct orifices.[12]

Thus, one explanation for the discrepancy between 5-9 and 15-20 ducts may well be the sebaceous glands/tubercles/tubes that mimic the appearance of ducts behind the nipple but do not contribute to the ductal lobular infrastructure of the breast. An additional possibility is that some ducts bifurcate shortly after emerging from the nipple. Teboul[7] described his findings in his series of over 6000 breasts studied through ultrasound and ductoscopy. He also describes 5-8 "milk pores" but suggests that some ducts join together behind the areola to form common collectors. In our observations of nipple ductal orifices in lactating women we also found an average of 5-9 openings in the nipple. This was further confirmed in the postmastectomy specimens where all of the nipple ductal orifices were cannulated and the underlying ducts demonstrated to contain dye, thus confirming the findings of Cooper, Sartorius and Teboul.[5-7] Interestingly, in all of the studies which accessed the nipple duct orifices from above, there was general agreement with the finding of 5-9 ductal orifices. All of the studies that used surgical specimens and identified the ducts from below the nipple, however, suggested 15-20 orifices. This directional discrepancy may be due to either misidentifying the additional "sebaceous glands"/tubercles/tubes as breast ducts or incorrectly recognizing a lobe

as being completely distinct as described by Teboul.[7] This misinterpretation may well explain the discrepancy in the data such as that of Ohtake *et al.*[8] who used nipple sparing quadrantectomies to do elegant 3-D computer reconstructions of the mammary duct-lobular systems. The authors in that study found 16 mammary ductal systems in one breast and four with intraductal anastomoses. It is possible, however, that the ducts with anastomoses were in fact part of the same ductal system uniting with each other with one of Teboul's "common collectors" behind the nipple. Moffat and Going[9] used subgross sections from the autopsy of a 19 year old girl. They were able to trace ten ducts completely which they felt represented half of the ductal systems present. Since they did not start at the nipple orifices, they could also be observing the 15-20 hollow ducts identified by Teboul[7] or the sebaceous glands described by Sartorius[6] rather than distinct and independent ductal systems.

Another point of controversy alluded to briefly in the Ohtake study[8] is whether the ductal systems really anastomose with each other. Cooper[5] stated definitively that they did not. Interestingly, in our present study conducted 158 years later, we used a combination of colored dyes to support the same hypothesis. Cooper did, however, describe an intertwining of the ductal systems like the roots of a tree. It is this intertwining which makes it impossible to differentiate ductal systems on routine histopathology and may also account for the confusion about the

number of ductal systems. In our early ductoscopy studies[2] and in our present work we note that adjacent ductal profiles often belonged to different ductal systems. Moffat and Going,[9] in their computer model of the ductal "catchment areas," were able to elegantly show the overlapping of ductal systems. The assumption that adjacent ductal profiles are part of the same ductal system may be the source of erroneous conclusions about multicentricity in precancerous disease processes such as DCIS and in the adequacy of surgical resection margins. Even research studies looking for genetic markers such as loss of heterozygosity (LOH) in breast tissue adjacent to cancer may not be sampling the same ductal system. We found no evidence of ductal anastomoses in our studies. If anastomoses do occur, they are probably rare.

In most of the previous studies the ductal systems have been described as radial. This may well result from the fact that most of the examinations have been done on breasts that have been removed and processed histopathologically and therefore two dimensionally. Cooper[5] stated that, some ducts radiate from the nipple while others pass directly backwards to the posterior surface of the gland. Teboul[7] reported a radial distribution from his ultrasound studies. His technique involved identifying a duct near the nipple and following it with real time ultrasound. This would necessitate compressing the breast toward one side or another, which would not be conducive to placing each ductal

system in three-dimensional space. Sartorius[6] developed a classification system for the distribution of the ducts to analyze all of his ductograms according to this scheme (unpublished data). He described one duct which was central which projected directly back to the chest wall. He also described central lateral and central medial ductal systems which projected back on either side of the central duct. In addition, there were central upper and central lower. It is not clear from his studies whether he felt that there were some ductal systems or lobes, as he called them, that projected radially. Suffice it to say there are at least some indications from Cooper[5] and possibly Sartorius[6] that support our conclusions regarding our independent analysis of the Sartorius' ductograms.

We realize that the results of our retrospective analysis of the Sartorius ductograms have to be interpreted carefully because there were many inherent biases in this type of analysis. First of all there was no attempt by Sartorius to catheterize a particular duct but rather only the duct which was most apparent. The resulting ductograms would be expected to be biased toward the ductal orifices which were more obvious or produced NAF. It is possible, then, that some of the ductal systems would not be represented in his series at all. Second, there would be no standardization of technique. The ductograms were done by the same technician, but there was no standardization of positioning of the breast. This meant that a ductal system which appeared to be lateral in one

woman could actually have been central in another. Taking all this into account, we still felt that we could mine these ductograms for useful information --- that a detailed retrospective anatomical analysis of this series of ductograms could give us a general idea of the pattern and number of ductal systems. Using this retrospective analysis, even with its limitations, we find support for our conclusions based on our other approaches (#1-#3) that the ductal systems and ductal orifices are either central or peripheral. The central ducts project toward the chest wall and the peripheral ones drape around them.

Why is knowledge of ductal anatomy important? The nipple ductal orifice is the entry to the ductal lobular unit of the breast. With growing acknowledgement that breast cancer occurs in one ductal system[13] we need to consider the breast as a collection of 6-8 ductal systems. Preliminary studies indicate that hormone levels differs in different ductal systems[14] and it may well be that each ductal system represents a different microenvironment? Ductoscopy, lavage, cytology and pathology correlation studies are presently evolving to address this question.[15]

Meanwhile what we have described has many immediate practical clinical applications. The most obvious is in the planning of surgery. In our Chilean study, we were able to use a mammogram to predict which nipple ductal orifice communicated with the lesion, identify the orifice with transareolar dye and confirm the connection with a ductogram. This technique could be therefore be used to map the ductal system

preoperatively with select ductograms through the relevant nipple orifices and allow a more precise surgical procedure. Since DCIS and invasive carcinoma seem to emanate from a single duct even in women with a genetic predisposition (ie., BCRA1, BCRA2) to the disease, that specific duct may express a surrogate marker before there is any histologic abnormalities. Identification of a surrogate marker either in ductal fluid or cells obtained by ductal lavage will identify the high risk duct rather than just the high risk patient. Accessing the right duct and the right orifice would be critical in obtaining this information. A knowledge of ductal orifice anatomy allows for a separation of central from peripheral ducts, a separation of NAF ducts from non-NAF ducts, a separation of immediately dye-labeled ducts from delayed-labeled ducts all of which would foster comparative studies of surrogate markers. If a ductal system at risk could be identified, specific ductal chemopreventive studies could be initiated. Directed ductoscopy, ductal lavage and future intraductal therapy then all would be predicated on the knowledge of the correct anatomy of the ductal system.

Contributors

Sanford Barsky drafted the report, participated in the research and approved the final version of the paper

Susan Love conceived the study, participated in the research and analysis and wrote the paper.

Conflict of interest statement

No direct conflicts of interest. Susan Love MD founded ProDuct Health which commercialized the intraductal catheter. It was acquired in 2002 by Cytyc Health Inc. She is a consultant to and shareholder in Cytyc Health, Inc.

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Figures

Figure 1: A) Two way histogram showing relative frequency/numbers of the nipple openings by location. Darker shading indicates higher relative numbers in the region of the nipple. The most common location was in the center of the nipple.

B) Mean location and standard error of the mean location, on the coordinates defined as x and y of the nipple openings in each of the 13 empirical clusters.

C) Artist's depiction of the general location of nipple openings.

Figure 2: Serial sectioning of the nipple identified numerous isolated keratin plugs (A); many keratin plugs associated with sebaceous glands (arrow) (B); but only rare keratin plugs overlying a ductal orifice (C). Here an underlying duct courses upward (arrows) toward the nipple to emanate at the nipple ductal orifice. These underlying ducts could be highlighted by anti-cytokeratin immunocytochemistry (D).

Figure 3: The normal nipple exhibits a corrugated surface with numerous dimples and the ductal orifices are not readily visible (A); transarealor-injected dye initially wicks up into a central duct (B); and then additional and more peripheral ducts are identified (C). With this technique multiple nipple ductal orifices could be identified and cannulated with glide wires (D).

Figure 4: Instillation of water-insoluble dyes in cannulated nipple ductal orifices fill one entire ductal lobular unit (A), evidence that the approach can reach the deep recesses of the breast. Instillation of different colored dyes through different orifices is depicted (B). Although different ductal systems can appear in the same quadrant juxtaposed to one another there is no admixing of dyes and therefore no interductal communications.

Figure 5: A) Two-way histogram showing the relative frequency/concentration of the ducts, by ductal center location (x duct, y duct). Darker shading indicates higher relative numbers of ducts projected in that region of the chest wall.

B) Mean location and its standard error of each of the 11clusters resulting from an analysis of one ductogram per woman (selected at random) (n=474)

C) Mean location and its standard error of each of the 13 clusters identified by using only ductograms of women 50 years old or younger (n=220)

Figure 6: An artist's rendition of the ductal anatomy

Tables

CHAPTER 4

Maspin and Myoepithelial Cells

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CHAPTER 4

Maspin and Myoepithelial Cells

Sanford H. Barsky, Paul Kedeshian and Mary L. Alpaugh

Introduction

Host cellular paracrine regulation of tumor progression is an important determinant of tumor growth, invasion and metastasis but one cell which has largely been ignored in this regulation is the myoepithelial cell. In any organ where there is significant branching morphogenesis such as the breast, myoepithelial cells ubiquitously accompany and surround epithelial cells and are thought to keep in check (negatively regulate) the process of branching. Myoepithelial cells surround both normal ducts and precancerous lesions, especially of the breast (so-called DCIS, ductal carcinoma-in-situ), and form a natural border separating proliferating epithelial cells from proliferating endothelial cells (angiogenesis). Myoepithelial cells, by forming this natural border, are thought to negatively regulate tumor invasion and metastasis. Whereas epithelial cells are susceptible targets for transforming events leading to cancer, myoepithelial cells are resistant. Indeed tumors of myoepithelial cells are uncommon and when they do occur, are almost always benign. Therefore it can be said that myoepithelial cells function as both autocrine as well as paracrine tumor suppressors. Our laboratory has found that myoepithelial cells secrete a number of suppressor molecules including high amounts of diverse proteinase inhibitors which include TIMP-1, protease nexin-II, and α-1 antitrypsin, but low amounts of proteinases and high amounts of diverse angiogenic inhibitors which include thrombospondin-1 and soluble bFGF receptors but low amounts of angiogenic factors compared to common malignant cell lines. However the most striking difference between the suppressive effector molecules secreted by myoepithelial cells and carcinoma cells is the levels of maspin secretion. Whereas carcinoma cells do not secrete maspin, myoepithelial cells secrete this serpin in large quantities. This observation holds in vitro, in mice, and in humans and suggests that maspin and myoepithelial cells exert pleiotropic suppressive effects on tumor progression. Since maspin is both a proteinase inhibitor, a locomotion inhibitor and an angiogenesis inhibitor, the diverse actions of maspin may largely explain the pronounced anti-invasive and anti-angiogenic effects of myoepithelial cells on carcinoma and pre-carcinoma cells. The same actions of maspin also may account for the low grade biology of myoepithelial tumors which are devoid of appreciable angiogenesis and invasive behavior. Finally since maspin is a secretory product of myoepithelial cells, the presence of maspin in body fluids such as in breast ductal fluid and in saliva reflects the structural and functional integrity of the

Maspin, edited by Mary J.C. Hendrix.

ductal-lobular units of the mammary and salivary glands respectively. Maspin, in ductal fluid, may serve as a surrogate (intermediate) end point marker (SEM) to estimate the risk of DCIS progression to invasive cancer in the breast and alternatively, in saliva, may serve as a tumor marker to detect the presence of incipient myoepithelial tumors occurring within the salivary glands of the head and neck.

Review of Current Research

Studies of Maspin and Myoepithelial Cells

It has become clear that cancer cells come under the influence of important paracrine regulation from the host microenvironment.[1] Such host regulation may be as great a determinant of tumor cell behavior in vivo as the specific oncogenic or tumor suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves.[2,3] Both positive (fibroblast, myofibroblast and endothelial cell) and negative (tumor infiltrating lymphocyte and cytotoxic macrophage) cellular regulators exist which profoundly affect tumor cell behavior in vivo.[4,5] One host cell however, the myoepithelial cell, has escaped the paracrine onlooker's attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, is thought to contribute largely to both the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and differentiating epithelial cells in health and to abnormally proliferating and differentiating epithelial cells in precancerous disease states such as ductal carcinoma in situ (DCIS) of the breast. This anatomical relationship suggests that myoepithelial cells may exert important paracrine effects on normal glandular epithelium and may regulate the progression of DCIS to invasive breast cancer. Circumstantial evidence suggests that the myoepithelial cell naturally exhibits a tumor suppressor phenotype. Myoepithelial cells rarely transform and when they do generally give rise to benign neoplasms that accumulate rather than degrade extracellular matrix.[6] Myoepithelial cells directly or indirectly through their production of extracellular matrix and proteinase inhibitors including maspin are thought to regulate branching morphogenesis that occurs in the developing breast and salivary gland during embryological development.[7] There have been a paucity of studies on myoepithelial cells because they have been relatively difficult to culture and because tumors that arise from these cells and rare.

In previous studies we have been extremely fortunate to have successfully established immortalized cell lines and transplantable xenografts from benign or low grade human myoepitheliomas of the salivary gland and breast.[8,9] These cell lines and xenografts displayed an essentially normal diploid karyotype and expressed identical myoepithelial markers as their in situ counterparts including high constitutive expression of maspin. Unlike the vast majority of human tumor cell lines and xenografts which exhibited matrix-degrading properties, these myoepithelial lines/xenografts like their myoepithelial counterparts in situ retained the ability to secrete and accumulate an abundant extracellular matrix composed of both basement membrane and non-basement membrane components. When grown as a monolayer one prototype myoepithelial cell line, HMS-1, exerted profound and specific effects on normal epithelial and primary carcinoma morphogenesis.[8] These studies support our position

that our established myoepithelial lines/xenografts recapitulate a normal differentiated myoepithelial phenotype and can therefore be used experimentally as a primary myoepithelial surrogate. Prompted by these studies and by the conspicuous absence of studies examining the role of the myoepithelial cell in tumor progression, we decided to examine the myoepithelial cell from this perspective. Experiments with these cell lines/xenografts together with relevant in situ observations form the cornerstone of our studies which observe that the human myoepithelial cell is a natural tumor suppressor.

Maspin and Myoepithelial Cells Inhibit Tumor Invasion

Breast ducts and acini are surrounded by a circumferential layer of myoepithelial cells exhibiting strong immunoreactivity for S100, smooth muscle actin, and diverse proteinase inhibitors including maspin, α1-AT, PNII/APP, and TIMP-1 (Fig.1A). In DCIS, the myoepithelial layer appeared either intact or focally disrupted, but the myoepithelial cells themselves exhibited the same pattern of immunoreactivity (Fig. 1B). In DCIS although proliferations of vWf immunoreactive blood vessel capillaries were observed focally within the supporting stroma, such blood vessels were not observed within the proliferating DCIS cells on the epithelial side of the myoepithelial layer (Fig.1C). The human tumoral-nude mouse xenografts derived from the human myoepitheliomas of the salivary gland, HMS-X and HMS-3X, and breast, HMS-4X demonstrated an immunocytochemical profile identical to each other and to that exhibited by the myoepithelial cells surrounding normal ducts and DCIS with especially intense maspin immunoreactivity (Fig.1D). Not only was strong proteinase inhibitor immunoreactivity present within the myoepithelial cells of these xenografts, but strong proteinase inhibitor immunoreactity could be demonstrated within their extracellular matrix as well. Within this abundant extracellular matrix deposited by the different human myoepithelial xenografts, murine blood vessels were not observed (Fig. 1E, Fig.1F; Fig 1G). Using a mouse specific Cot-1 DNA probe (Fig. 1H), human myoepithelial xenografts HMS-X, HMS-3X, and HMS-4X, demonstrated an absent or near absent murine component indicative of absent to near absent angiogenesis. Human non-myoepithelial xenografts of breast cancer cell lines, MDA-MB-231 and MDA-MB-468, in contrast, showed a comparatively large murine component of presumed angiogenesis. The human myoepithelial xenografts, HMS-X, HMS-3X, and HMS-4X, instead exhibited a very dense accumulation of extracellular matrix accounting for a gross "pearl" appearance of these xenografts (Fig. 1I)

Detailed studies[9] conducted with HMS-1 (Fig. 1J), a prototype myoepithelial cell line, revealed a constitutively high proteinase inhibitor to proteinase ratio in strong contrast to the high proteinase to proteinase inhibitor ratio observed in a number of malignant human cell lines (Fig. 2A). Marker studies with this cell line and corresponding xenograft (HMS-X) reflected the constitutive gene expression profile of myoepithelial cells in situ (Fig. 2B). This was especially true with respect to maspin. Direct gelatin zymography of CM revealed only low levels of the 92 and 72 kDa type IV collagenases (MMP-9 and MMP-2 respectively) in HMS-1; the 72 kDa collagenase was reduced 6-fold in HMS-1 compared to the levels in the majority of the malignant lines; direct fibrin zymography revealed visibly lower levels of the 54 kDa urokinase plasminogen activator (uPA) in HMS-1. This was also observed in casein/plasminogen gels. Tissue type plasminogen activator was not detected in any cell line, nor was plasmin detected in control gels lacking plasminogen. Stromelysin-1 (MMP-3) was

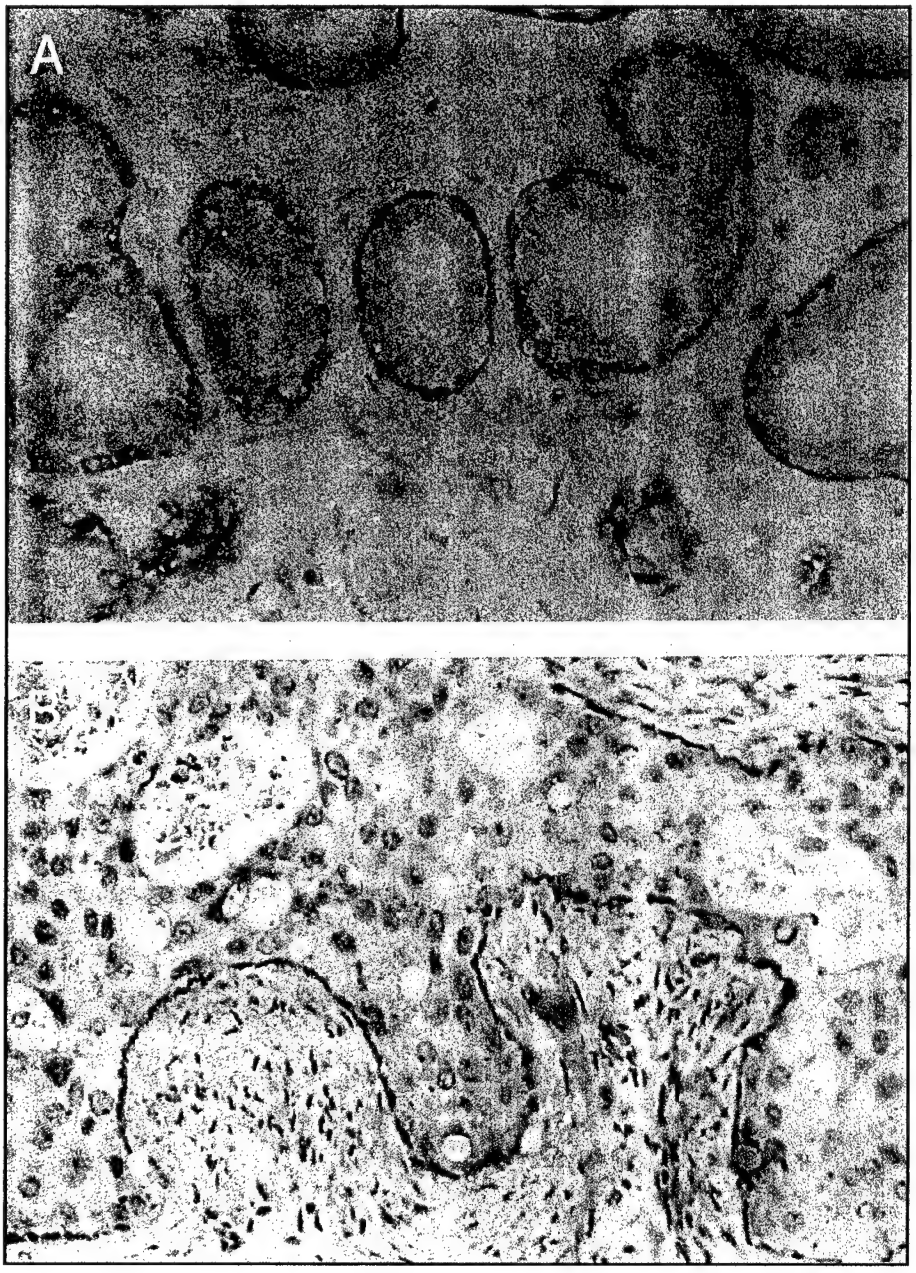

Fig. 1A & B. In situ immunocytochemistry profile of myoepithelial cells and their derived cell lines/ xenografts. (A) Differential maspin immunoreactivity of myoepithelial cells surrounding breast ducts and acini; (B) Differential maspin immunoreactivity of myoepithelial cells in DCIS.

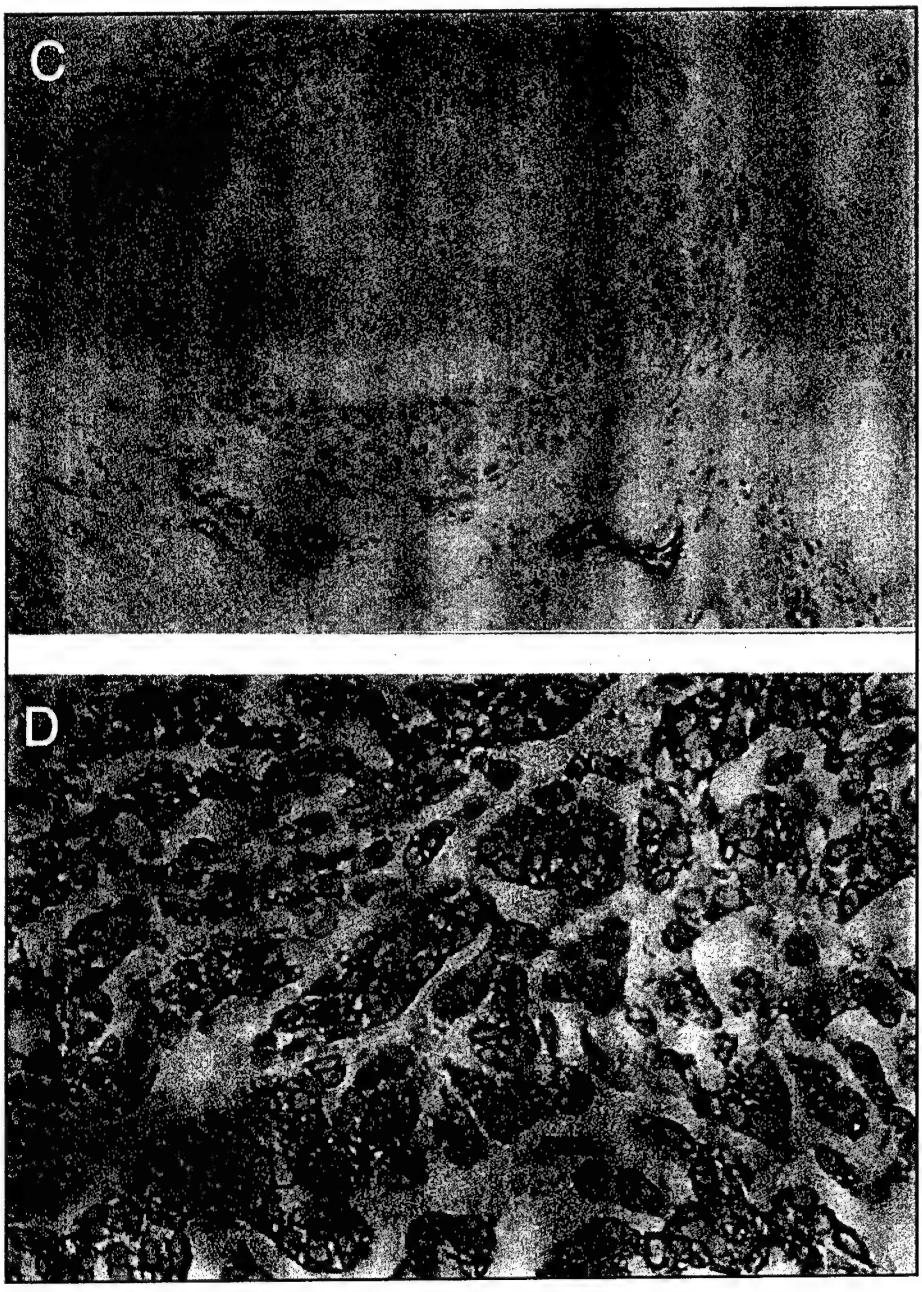

Fig. 1C & D. In situ immunocytochemistry profile of myoepithelial cells and their derived cell lines/ xenografts. (C) Angiogenesis demonstrated by vWf immunoreactivity limited to stromal side of DCIS; (D) Cytoplasmic maspin immunoreactivity of myoepithelial xenograft, HMS-X.

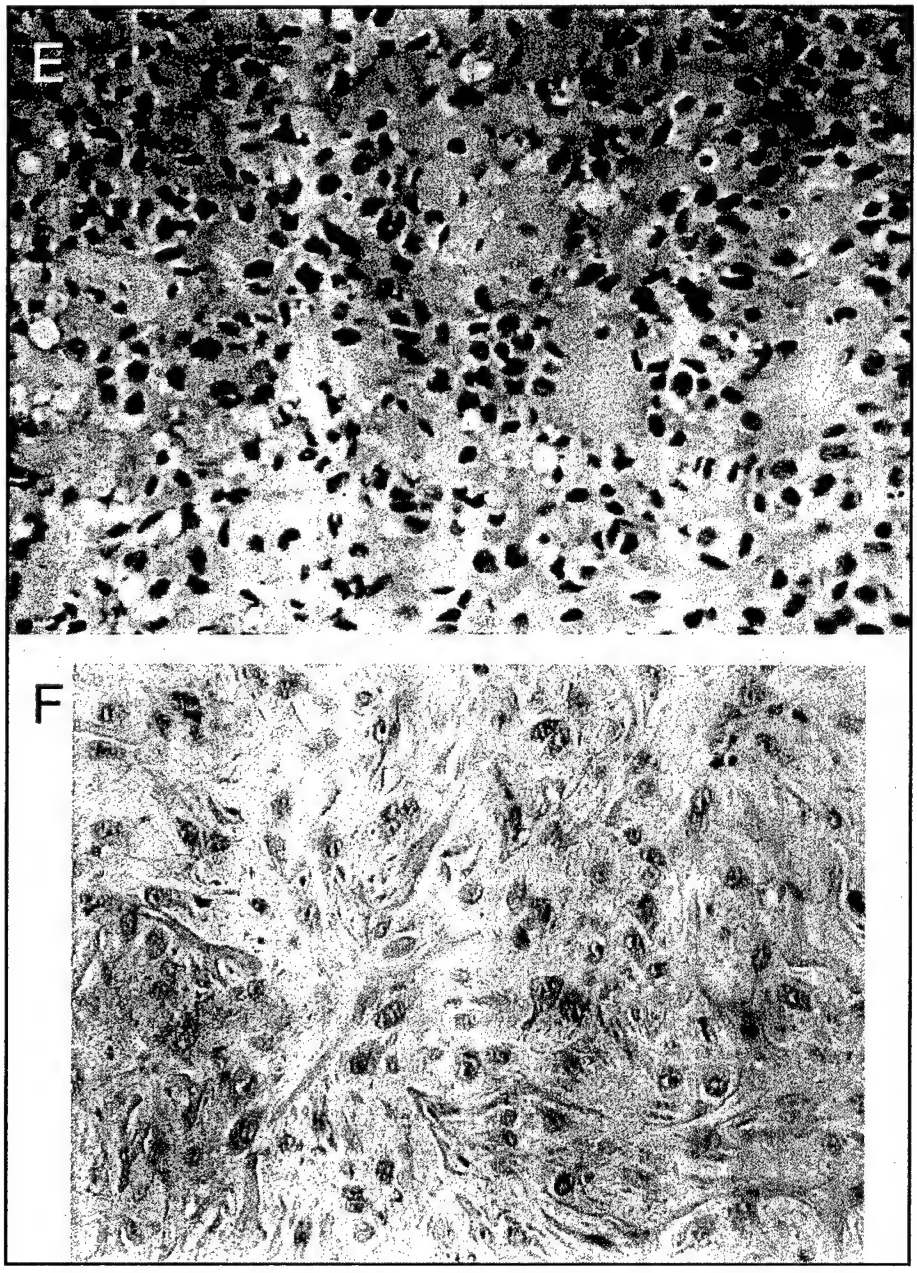

Fig. 1E & F. In situ immunocytochemistry profile of myoepithelial cells and their derived cell lines/ xenografts. (E) HMS-X, (F) HMS-4X.

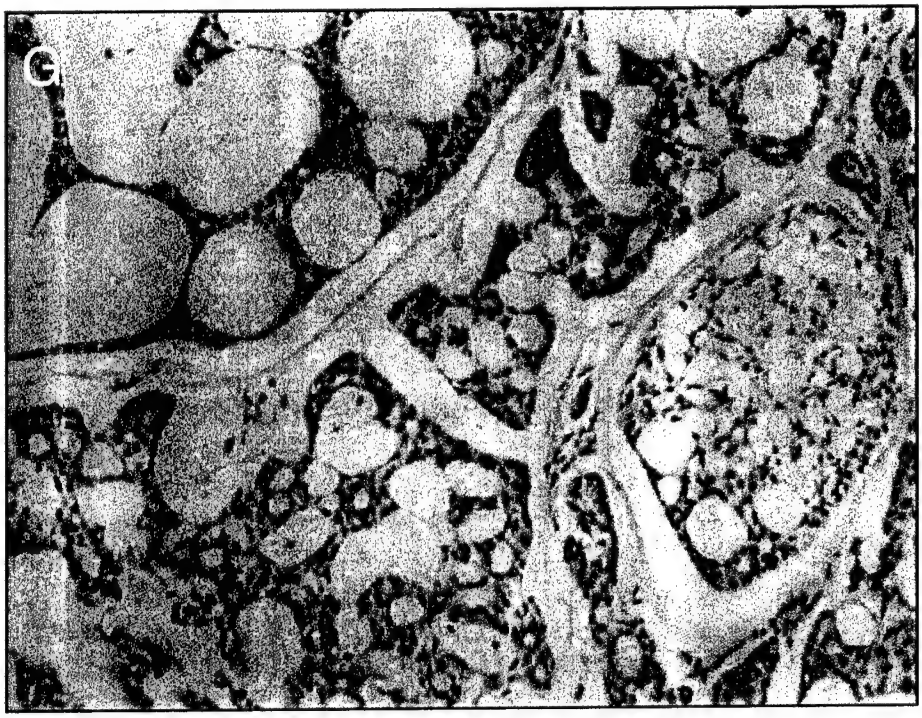

Fig. 1G. In situ immunocytochemistry profile of myoepithelial cells and their derived cell lines/ xenografts. HMS-6X myoepithelial xenografts exhibit abundant matrix accumulation devoid of apparent angiogenesis.

also not detected in HMS-1. The proteinase inhibitor expression profile of HMS-1, in contrast, was characterized by high constitutive expression in CM of several proteinase inhibitors including TIMP-1; PAI-1; three trypsin inhibitors: α1-AT, PNII/APP, and an unidentified 31 kDa inhibitor detected initially on reverse zymography; and the tumor suppressor maspin. With respect to the trypsin serine proteinase inhibitors, the conspicuous doublet at 116 kDa consistently greater in HMS-1 than in any of the other lines examined was confirmed on Western blot to be PNII/APP. These bands represented the 770 and 751 amino acid isoforms of PNII/APP which possessed a Kunitz-type serine proteinase inhibitor domain. Interestingly in 2M urea extracts of HMS-X, HMS-3X, and HMS-4X, a novel 95 kDa band of trypsin inhibition was detected by reverse zymography and confirmed by Western blot to represent an active breakdown product of PNII. This 95 kDa PNII breakdown product was completely absent from HMS-1 CM and urea extracts of HMS-1 cells suggesting that it was produced in situ within the myoepithelial extracellular matrix to which it bound. The retention of proteinase inhibitor activity by this breakdown product indicated that it retained the Kunitz-type serine proteinase inhibitor domain responsible for its ability to inhibit trypsin. In contrast to PNII/APP, protease nexin I was not detected. The second trypsin serine proteinase inhibitor was present at 54 kDa and was α1-AT. This inhibitor appeared nearly equivalent in HMS-1 compared to the malignant lines examined on reverse zymography, but by Western blot its signal was markedly stronger and slightly more mobile in HMS-1 than in the malignant lines. This data was

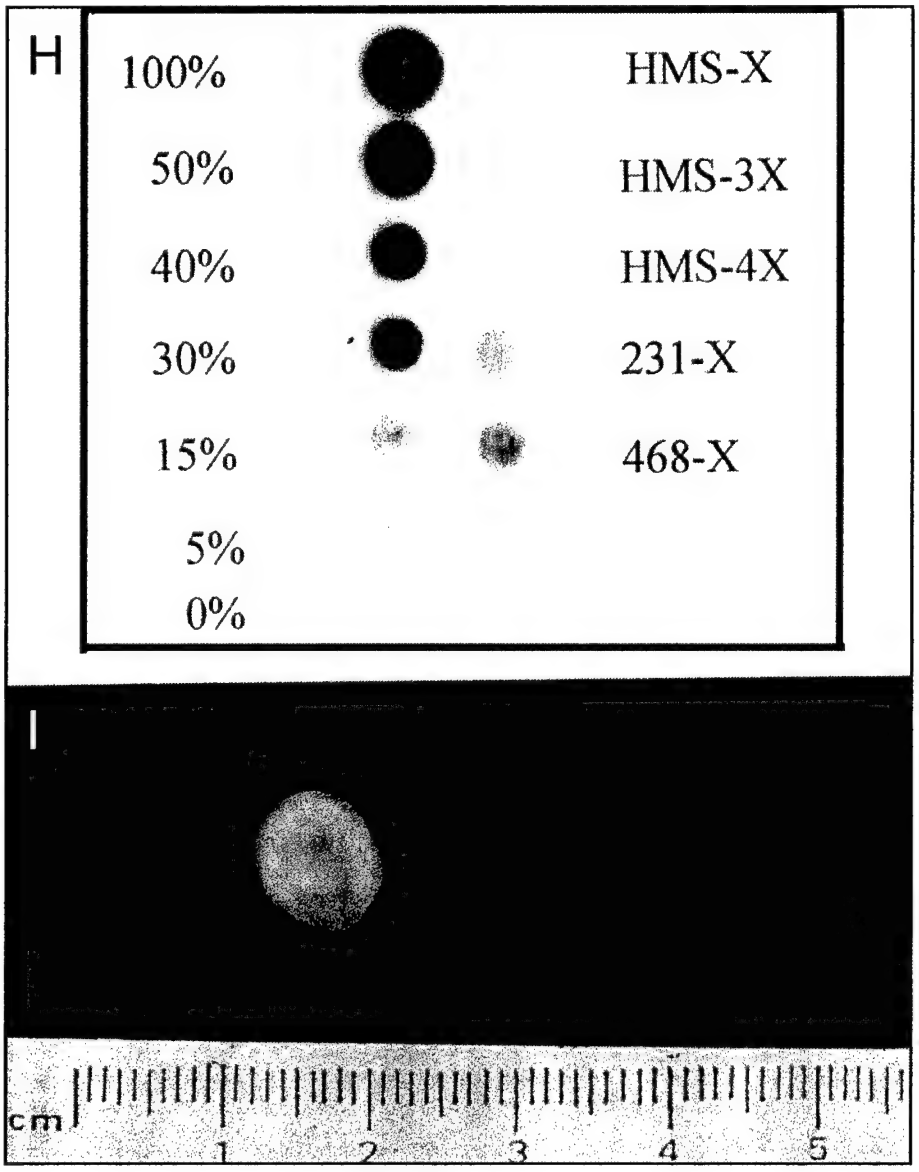

Fig. 1H & I. In situ immunocytochemistry profile of myoepithelial cells and their derived cell lines/ xenografts. (H) Murine Cot-1 dot blot. Using a mouse specific Cot-1 DNA probe, human myoepithelial xenografts, HMS-X, HMS-3X and HMS-4X, are devoid of a murine DNA angiogenic component in contrast to the angiogenic-rich MDA-MB-231-X and MDA-MB-468-X breast carcinoma xenografts (right column); control dot blots of varying murine DNA percentages are also depicted (left column); (I) The typical myoepithelial xenograft looks and shells out like a white glistening "pearl".

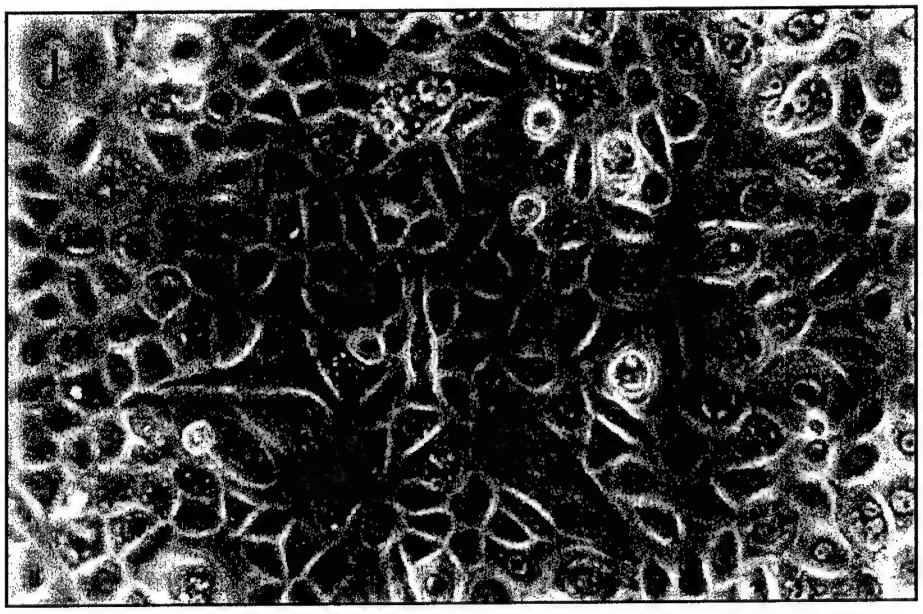

Fig. 1J. In situ immunocytochemistry profile of myoepithelial cells and their derived cell lines/ xenografts. Myoepithelial cells (HMS-1) grow as a monolayer in cell culture.

reconciled with the fact that α1-AT was probably less glycosylated in HMS-1. This relative underglycosylation caused α1-AT from HMS-1 to migrate slightly further into the gel and accounted for its poorer reactivation following SDS-denaturation on reverse zymography as compared to the more highly glycosylated isoforms present in the malignant lines. The third trypsin serine proteinase inhibitor detected at 31 kDa was clearly not a degradation product of either PNII or α1-AT as demonstrated by negative Western blot. The 31 kDa inhibitor was strongly expressed in HMS-1 and was either absent or nearly absent in all of the malignant lines examined. Whether this unidentified inhibitor is a novel inhibitor is being determined. In contrast to the above inhibitors, PAI-1 was expressed only slightly greater in HMS-1 compared to the majority of the malignant lines by both reverse zymographic and Western blot analysis. Neither PAI-2, PAI-3 or α2-antiplasmin were detected by Western blot analysis in any of the cell lines. Antiplasmin activity as determined by photometric assay was completely absent as well. The most striking difference, however, between the strong proteinase inhibitor profile of HMS-1 and the profile of the malignant cell lines examined was in the expression of maspin. Intense maspin transcripts (3.0 and 1.6 kb) and protein (42 kDa) were identified in HMS-1 and HMS-1 CM respectively but were completely absent in all of the malignant lines examined (Fig. 2A). With its proteinase inhibitor profile of increased maspin, TIMP-1, PNII, α1-AT and the 31 kDa inhibitor, HMS-1 bore strong resemblance to normal human mammary epithelial cells (HMEC) (Fig. 2A) except that the expression of all of these proteinase inhibitors including maspin was even more enhanced in HMS-1. Being derived from normal ducts and acini of the human breast, HMEC cultures likely contain myoepithelial as well as epithelial cells. Thus the resemblance of HMS-1 to HMEC further supported our contention that HMS-1, though immortal, expressed a well-differentiated myo-

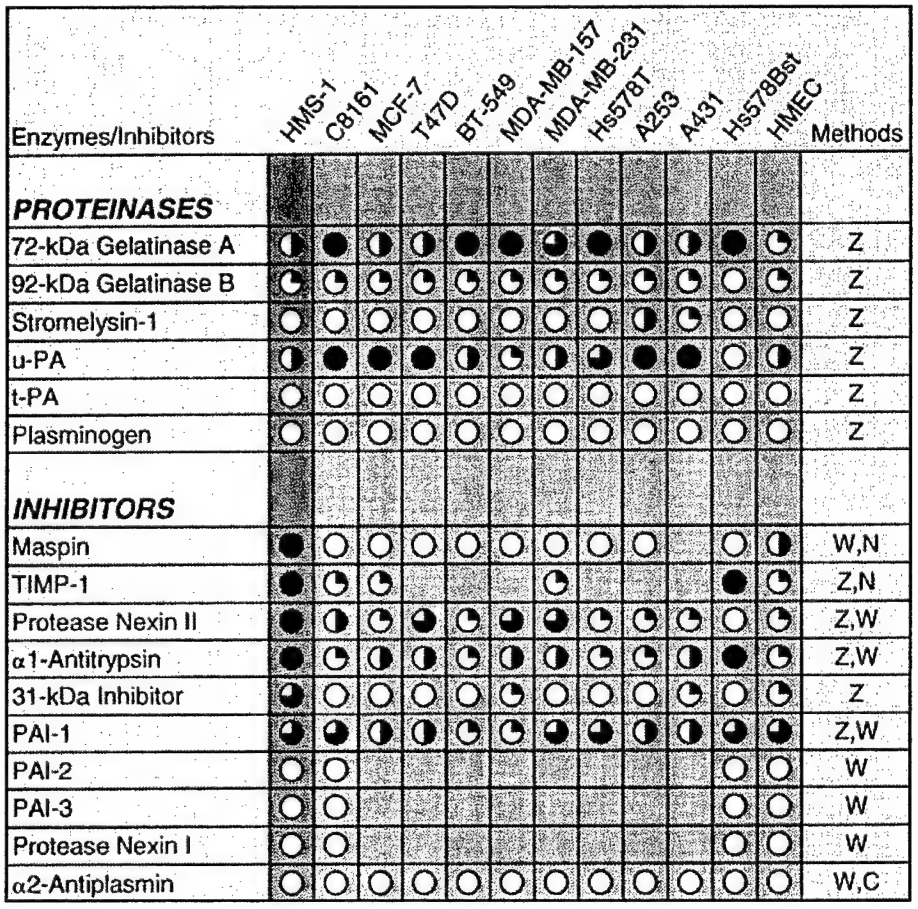

Fig. 2A. Relative constitutive expression of diverse proteinase inhibitors and proteinases in myoepithelial cells (HMS-1) compared to various malignant cell lines. Z, direct or reverse zymography; W, western blot; N, northern blot; C, chromogenic substrate assay.

epithelial phenotype. In addition, since HMS-1 was a clonal line expressing a pure myoepithelial phenotype, it would be predicted to express certain myoepithelial-associated proteins such as maspin, α1-AT, PNII/APP, and TIMP-1 to a greater degree than HMEC. Predictably, the myofibroblast line, Hs578Bst, was strongly expressive of TIMP-1 but did not express maspin, PNII or the 31 kDa inhibitor (Fig 2A). The strong proteinase inhibitor profile exhibited by HMS-1 was shared by all of the myoepithelial xenografts including HMS-X, HMS-3X, and HMS-4X.

In the modified Matrigel invasion chamber used in this study, HMS-1 cells and their conditioned media (CM) dramatically inhibited invasion of four invasive breast carcinoma cell lines (Fig. 2C). The HMS-1 line was itself non-invasive in this chamber. Predictably, the anti-invasive effects of HMS-1 could be abolished by CHX (40 μg/ml) 24 hr pretreatment. HMS-1 CM inhibited invasion in a dose response fashion up to 30%±8% of control ($p<.01$). Pretreatment of HMS-1 with dexamethasone (.25 μM) produced a complete invasion-permissive phenotype (100% of control) whereas

	HMS-X, 3X, 4X		Normal Breast		DCIS	
	Cells	Matrix	ME*	Epi‡	ME	Epi
S-100	++++§	-	++++	-	++++	-
Maspin	++++	-	++++	+	++++	±
α1-AT	++	++	++	-	++	-
PNH	++	+++	++	-	++	±
TIMP-1	++	+	++	-	++	-
PAI-1	+	±	+	+	+	+

Fig. 2B. Myoepithelial-related immunoreactivity in situ.
* myoepithelial cells; ‡ epithelial cells; § ++++, intensely positive; +++, strongly positive;++, positive; +, weakly positive; ±, equivocally positive; -, negative.

pretreatment with PMA (5 μM) produced an essentially nonpermissive phenotype (2% of control) ($p<.05$) (Fig. 2D). The effects of dexamethasone and PMA were quite dramatic. The effects of other agents including RA, dB-cAMP, Na-But, and 5-AzaC showed either permissive or non-permissive trends but were less dramatic. PMA's induction of the nonpermissive phenotype began after 20 minutes pretreatment, was almost complete after 2 hr and maximized after 24 hr ($p<.05$). The induction of this nonpermissive phenotype correlated with the induction of a dramatic 5-fold increase in maspin secretion measured in HMS-1 CM (Fig. 2E). As a result of PMA treatment, both an immediate release (within 2 minutes) of maspin from HMS-1 cells occurred (Fig. 2E) as well as a more sustained secretion for at least 24 hr following PMA pretreatment (Fig. 2E). The increased maspin secretion was not on the basis of an increase in steady state maspin transcripts (Fig. 2E). PMA also resulted in a less dramatic 2-fold increase in both MMP-9 and TIMP-1 secretion. Dexamethasone's induction of an invasion-permissive phenotype in HMS-1 was not associated with a change in either maspin transcription or secretion. Immunoprecipitation with anti-maspin antibody at 1/100 dilution successfully removed all detectable maspin from myoepithelial cell CM (Fig. 2F). This CM lost its ability to inhibit invasion (Fig. 2F). Similar results were observed with the CM from the other myoepithelial lines (HMS-3 and HMS-4) studied. None of the non-myoepithelial cell CM inhibited invasion.

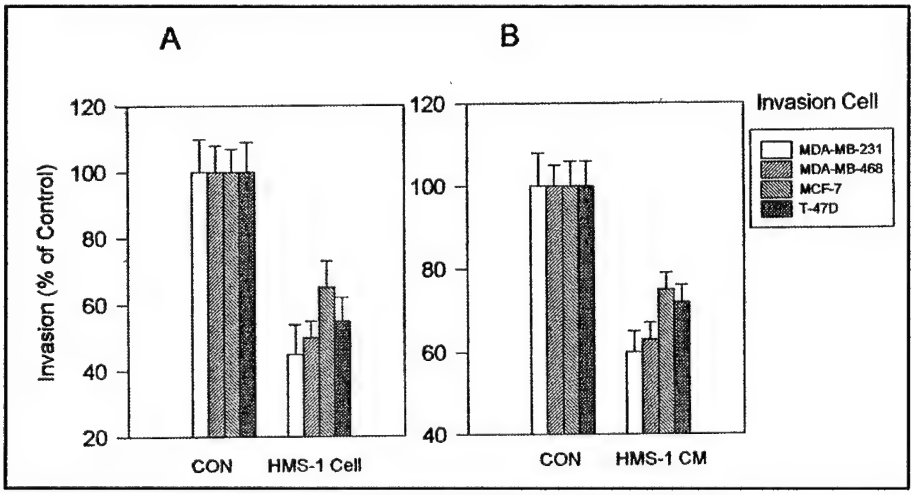

Fig. 2C. Effects of HMS-1 cells [A] and conditioned media (CM) [B] on MCF-7, T47D, MDA-MB-468 and MDA-MB-231 invasion. Both assays were performed in quadruplicate and show mean % invasion ± standard deviation.

Maspin and Myoepithelial Cells Inhibit Tumor Angiogenesis

Human myoepithelial cells which surround ducts and acini of certain organs such as the breast form a natural border separating epithelial cells from stromal angiogenesis. Myoepithelial cell lines (HMS-1-6), derived from diverse benign myoepithelial tumors, all constitutively express high levels of active angiogenic inhibitors which include maspin, TIMP-1, thrombospondin-1 and soluble bFGF receptors but very low levels of angiogenic factors.[10] Maspin recently has been shown conclusively to be an angiogenesis inhibitor.[11] As expected, our myoepithelial cell lines inhibit endothelial cell chemotaxis and proliferation. These myoepithelial cell lines sense hypoxia, respond to low O_2 tension by increased HIF-1α but with only a minimal increase in VEGF and iNOS steady state mRNA levels. Their corresponding xenografts (HMS-X-6X) grow very slowly compared to their non-myoepithelial carcinomatous counterparts and accumulate an abundant extracellular matrix devoid of angiogenesis but containing bound angiogenic inhibitors. These myoepithelial xenografts exhibit only minimal hypoxia but extensive necrosis in comparison to their non-myoepithelial xenograft counterparts. These former xenografts inhibit local and systemic tumor-induced angiogenesis and metastasis presumably from their matrix-bound and released circulating angiogenic inhibitors. These observations collectively support the hypothesis that the human myoepithelial cell (even when transformed) is a natural suppressor of angiogenesis.

Myoepithelial cells in situ separate epithelial cells from stromal angiogenesis, and this seemingly banal observation serves to illustrate the fact that stromal angiogenesis never penetrates this myoepithelial barrier (Fig. 1C) raising the hypothesis that myoepithelial cells are natural suppressors of angiogenesis. This observation was reinforced by a microscopic, immunohistochemical and DNA analysis of our myoepithelial xenografts. Our diverse myoepithelial xenografts secrete and accumulate an abundant extracellular matrix which is devoid of blood vessels in routine hematoxylin

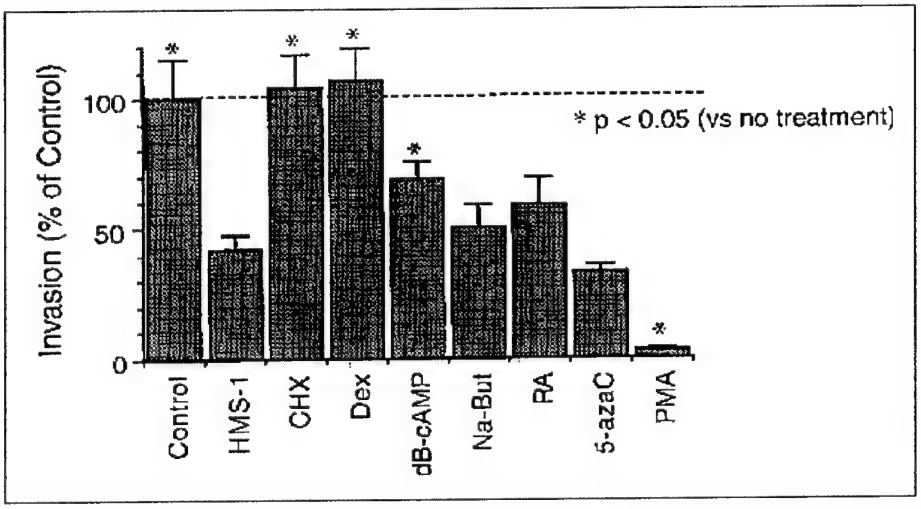

Fig. 2D. Effects of pharmacologic treatment of HMS-1 cells with various agents inducing permissive and non-permissive phenotypes: CHX, cyclohexamide; DEX, dexamethasone; dB-cAMP, N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate; Na-But, sodium butyrate; RA, all *trans* retinoic acid; 5-azaC, 5-azacytidine; PMA, phorbol 12-myristate 13-acetate.

and eosin staining (Fig. 1E; Fig. 1F; Fig. 1G) and vWf immunocytochemical staining in contrast to non-myoepithelial xenografts which show bursts of blood vessels. Quantitation of vessel density in 10 HPFs reveals absent to low vessel density in the myoepithelial xenografts compared to the non-myoepithelial xenografts ($p<0.01$). As mentioned previously, murine DNA Cot-1 analysis further reveals the absence of a murine component in the myoepithelial xenografts. Since in the xenografts, angiogenesis would be murine in origin, the absence of a murine DNA component is another indication that angiogenesis is minimal. Interestingly the myoepithelial xenografts grew slowly compared to the non-myoepithelial xenografts, a feature which was not found in comparisons between the myoepithelial *v* non-myoepithelial cell lines.

To explain these in vivo observations, we analyzed the gene expression profiles of our myoepithelial cell lines *v* non-myoepithelial cell lines with respect to known angiogenic inhibitors and angiogenic factors. HMS-1, as a prototype myoepithelial cell line, constitutively expressed none of the known angiogenic factors including bFGF, aFGF, angiogenin, TFGα, TGFβ, TNF-α, VEGF, PD-ECGF, PlGF, IFα, HGF, and HB-EGF but rather expressed maspin, thrombospondin-1, TIMP-1 and soluble bFGF receptors at high levels; this was in contrast to a high angiogenic factor (which included bFGF, VEGF, TFGα, TGFβ, HB-EGF, and PD-ECGF) to angiogenic inhibitor gene expression profile which was observed in non-myoepithelial cell lines. Other myoepithelial cell lines (HMS-2-6) exhibited an angiogenic inhibitor/angiogenic factor profile similar to that of HMS-1. Interestingly in 2M urea extracts of the myoepithelial xenografts but not in any of the non-myoepithelial xenografts, strong thrombospondin-1, TIMP-1 as well as plasminogen and prolactin fragments could be detected by Western blot. HMS-1 and HMS-1 CM (concentrated 10-100 fold) exerted a marked inhibition of endothelial migration and proliferation, both of which were abolished by pretreatment of the myoepithelial cells with cyclohexamide or dexamethasone. HMS-1 cells themselves did not migrate in response to either K-SFM,

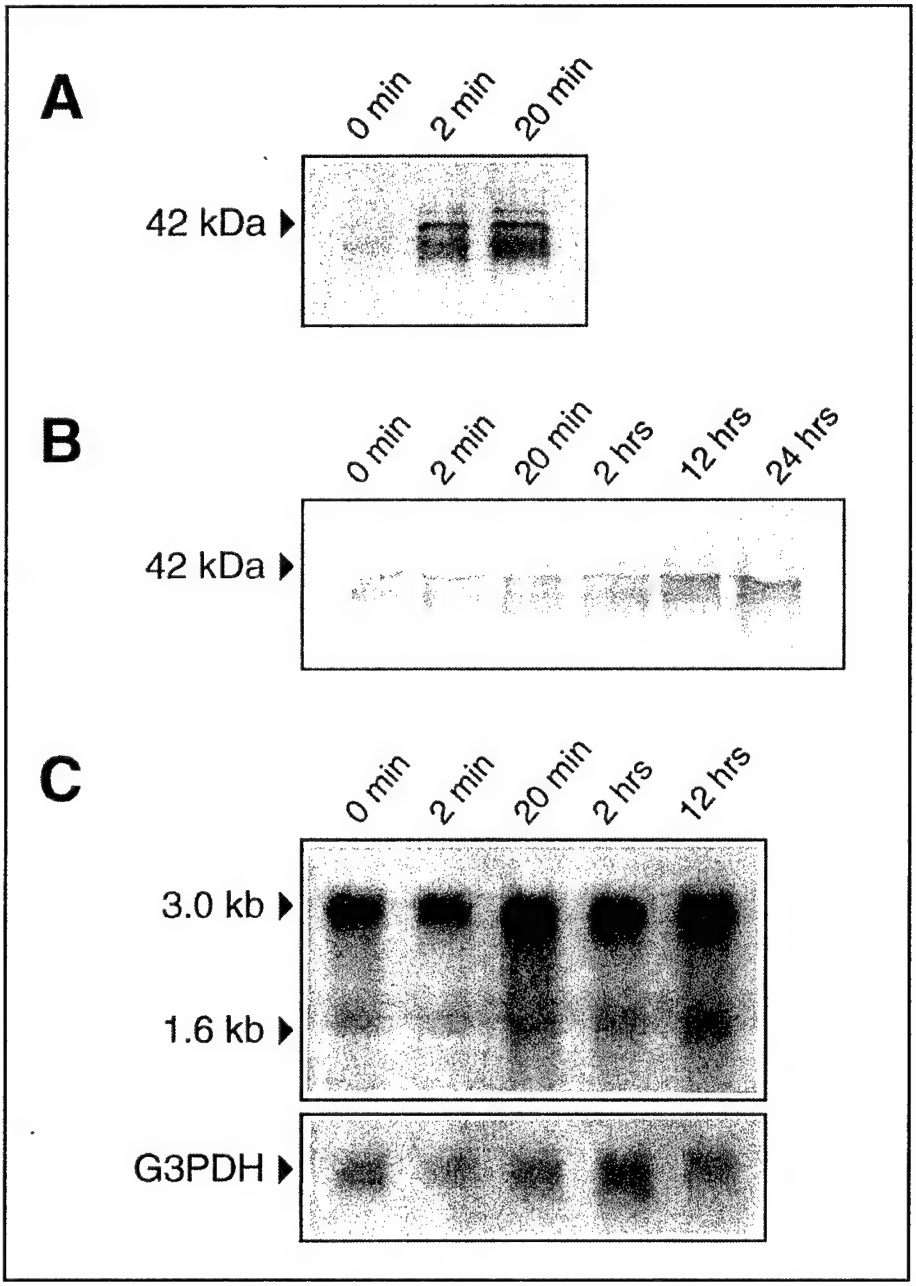

Fig. 2E. Immediate effects of PMA treatment on maspin secretion measured by Western blot of CM at designated times of PMA exposure [A]; delayed effects of PMA on maspin secretion measured by Western blot of CM 72 hours after PMA pretreatment for indicated time periods followed by removal of PMA [B]; Northern blot of maspin expression following exposure to PMA for indicated time periods [C].

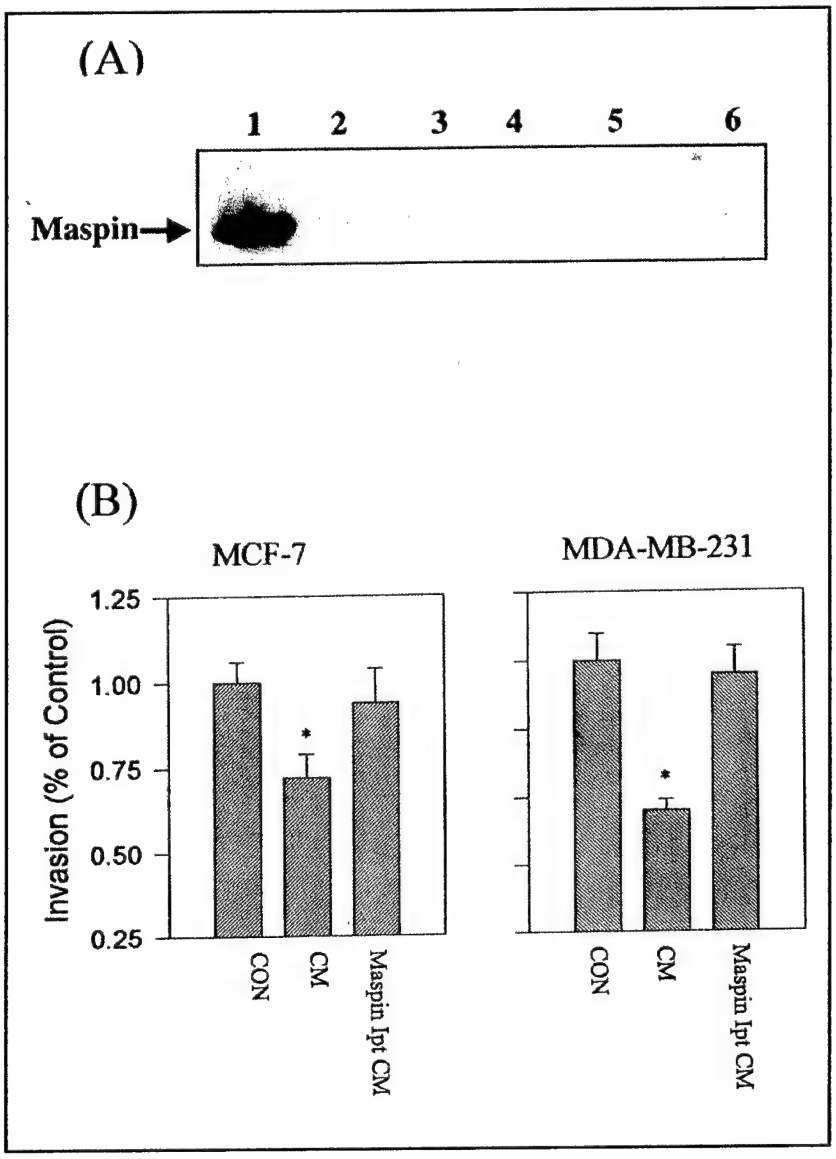

Fig. 2F. Maspin immunoprecipitation fraction [A] at various dilutions of maspin antibody (1/100), lane 1; (1/500), lane 2; (1/1000), lane 3; (1/2000), lane 4. Optimal dilution was 1/100 to achieve nearly 100% immunoprecipitation. Other serpin antibodies used including anti-PAI-1 (lane 5) and anti-PAI-2 (lane 6) resulted in only negligible cross-reacting immunoprecipitation of maspin. [B] Effects of HMS-1 25X CM and maspin-immunoprecipitated CM on breast carcinoma invasion. Control levels of invasion of designated breast carcinoma cell lines, MCF-7 and MDA-MB-231 were assigned arbitrary values of 1.0 and effects of CM and immunoprecipitated CM were expressed relative to these control levels. Results with other myoepithelial cell lines were similar. *indicates statistically significant differences compared to control ($p<0.05$).

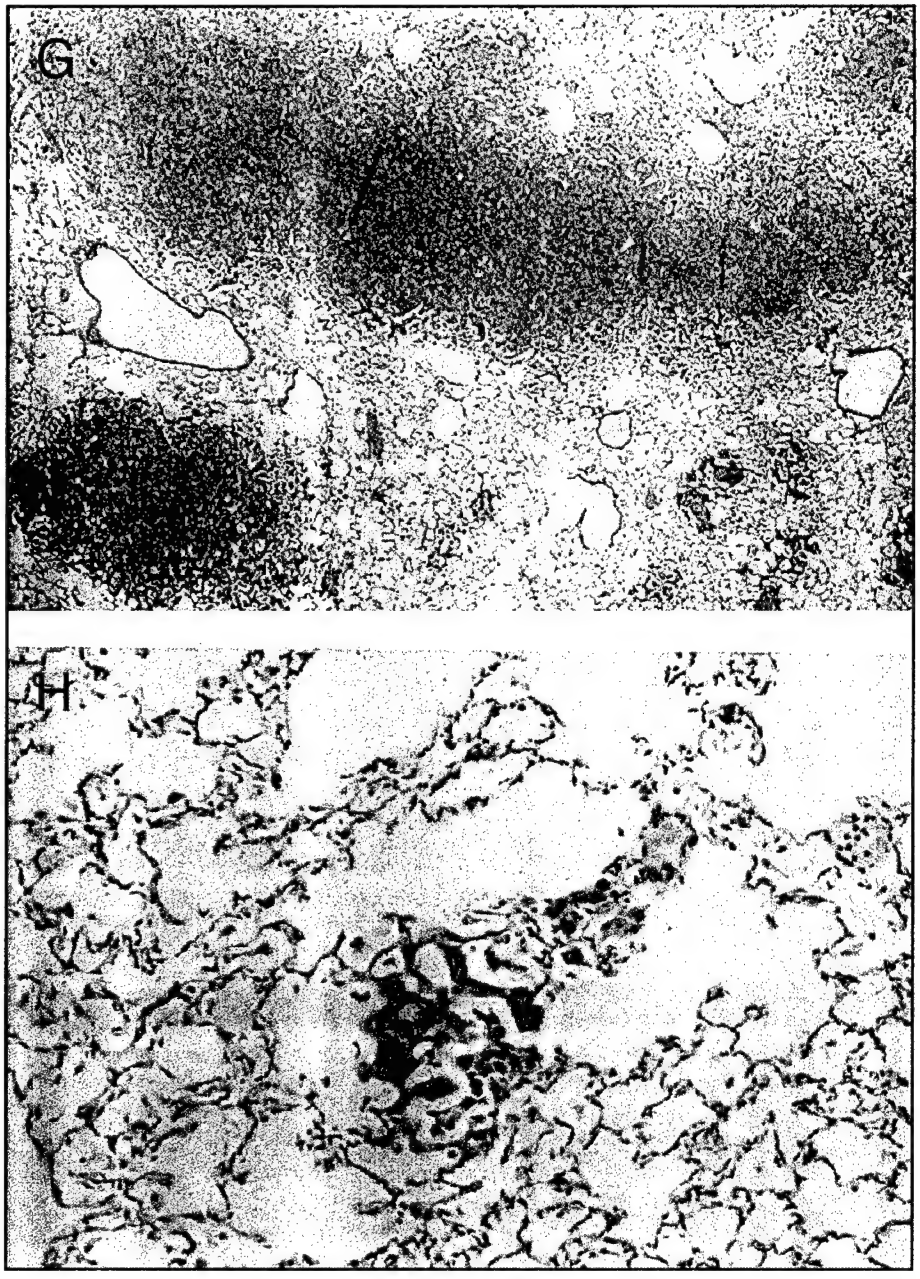

Fig. 2G & H. Differences in hematogenous pulmonary metastases with tail vein injected *neo*C8161 is in evidence in mice harboring non-myoepithelial xenografts (G) *v* myoepithelial xenografts (H). The number and size of metastatic colonies in mid-longitudinal cross section of lung was determined by digital image analysis and expressed as mean ± standard error.

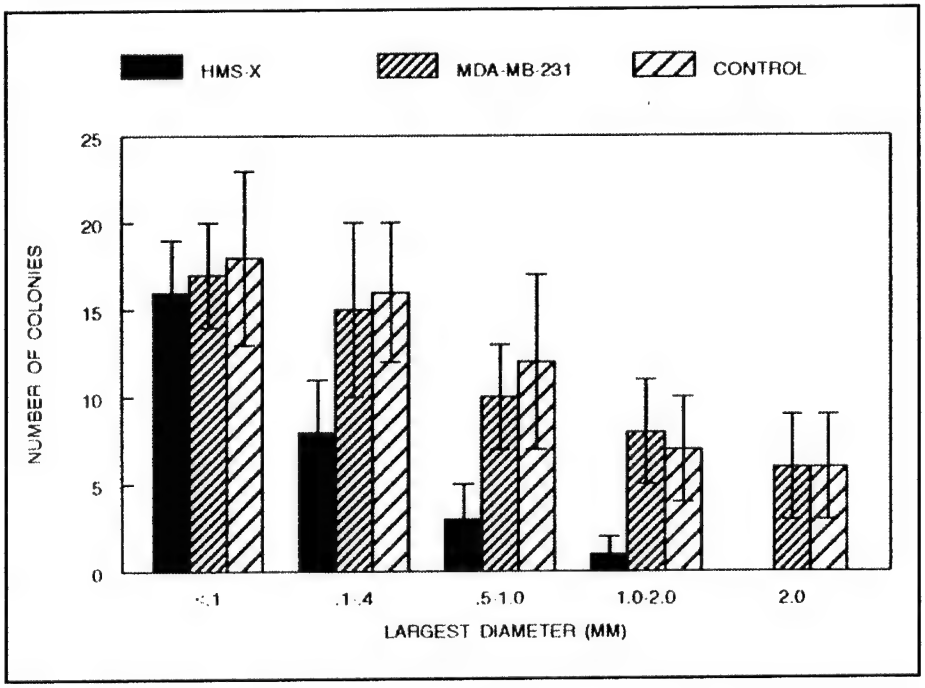

Fig. 21. Quantitation of pulmonary metastases revealed similar numbers of colonies in all three groups but a marked decrease in size in the group harboring the myoepithelial xenografts. Results depict a representative myoepithelial xenograft, HMS-X, a representative non-myoepithelial xenograft, MDA-MB-231, and control (no xenograft). Other myoepithelial and non-myoepithelial xenografts recapitulated these results.

FCS, or bFGF. When mixed with UVE, HMS-1 cells reduced endothelial migration to 12%±6% of control ($p<0.01$). HMS-1 concentrated CM reduced migration to 8%±7% of control ($p<0.01$). All of the non-myoepithelial malignant human cell lines studied stimulated both endothelial migration and proliferation. Concentrated CM from HMS-1, when fractionated on a heparin-Sepharose column, inhibited endothelial proliferation to 47%±10% of control ($p<0.01$). This inhibitory activity was present only in the 1.5-2.0M gradient fraction. Pretreatment of HMS-1 cells with PMA resulted in a 2-5 fold increase in endothelial antiproliferative inhibitory activity in both unfractionated CM as well as in the heparin-Sepharose fraction. Western blot of the heparin-Sepharose column fractions revealed that the 1.5-2.0M NaCl fraction contained thrombospondin-1. Immunoprecipitation of this fraction with anti-thrombospondin was effective at removing all thrombospondin-1 but decreased endothelial antiproliferative activity by only 50% raising the possibility that other angiogenic inhibitors including maspin were present in this fraction. The other myoepithelial cell lines (HMS-2-6) exhibited similar anti-angiogenic inhibitory activity in their fractionated and unfractionated CM. Therefore it is likely that both maspin and thrombospondin-1 are anti-angiogenesis effector molecules of myoepithelial cells.

To further explain our in vivo observations of minimal angiogenesis in our myoepithelial xenografts, in vitro and in vivo hypoxia studies were carried out. Non-myoepithelial xenografts, e.g., MDA-MB-231 exhibited florid hypoxia but only minimal

necrosis when they reached a size of 2.0 cm. In contrast, the myoepithelial xenografts exhibited only minimal hypoxia but prominent necrosis ($p<0.001$) at the same size of 2.0 cm. Quantitation of the areas of hypoxia (pimonidazole immunoreactivity) and areas of necrosis in the myoepithelial *v* non-myoepithelial xenografts suggested that in the myoepithelial tumors where angiogenesis is minimal hypoxic areas progress to necrosis rapidly whereas in the non-myoepithelial tumors hypoxic areas accumulate but do not progress to necrosis presumably from the angiogenesis which the hypoxia elicits. Comparative analysis of myoepithelial *v* non-myoepithelial cell lines to low O_2 tension revealed that while both cell lines sense hypoxia in that they responded by increasing HIF-1α, the myoepithelial lines upregulated their steady state mRNA levels of the downstream genes, VEGF and iNOS to a lesser extent than the carcinoma lines suggesting the possibility of decreased transactivation of HRE. Specifically we observed an approximate 1.7-fold increase in VEGF (1.1-fold increase in iNOS) in myoepithelial cells in response to hypoxia compared to an approximate 2.5-fold increase in VEGF (1.5-fold increase in iNOS) in carcinoma cell lines in response to hypoxia. Although these fold differences by themselves were not impressive, the absolute levels of VEGF (and iNOS) expressed in carcinoma cells in response to hypoxia were 2.5-fold greater for VEGF (and 1.7-fold greater for iNOS) than the levels of VEGF (and iNOS) expressed in myoepithelial cells in response to hypoxia. Therefore it can be concluded that myoepithelial cells did not express VEGF or iNOS in response to hypoxia to nearly the same extent as carcinoma cells. To study both local and systemic effects of myoepithelial cells on metastasis, spontaneously metastasizing tumor cells were injected into our myoepithelial xenografts. The highly metastatic *neo*C8161 cells injected into the myoepithelial xenografts could be recovered in significant numbers although the numbers of clones recovered were less than those recovered from the nonmyoepithelial xenografts. Histological analysis of the extirpated xenografts revealed *neo*C8161 cells actively invading through all of the nonmyoepithelial xenografts in contrast to the appearance the myoepithelial xenografts where the *neo*C8161 cells were confined to the immediate areas around the injection site. Pulmonary metastases of *neo*C8161 were completely absent in the myoepithelial xenograft-injected group whereas they were quite numerous in the nonmyoepithelial group ($p<0.001$). Analysis of extirpated myoepithelial xenografts containing injected *neo*C8161 cells contained no evidence of murine angiogenesis by either vWf immunocytochemical studies or murine DNA Cot-1 analysis whereas a similar analysis of extirpated *neo*C8161 injected-nonmyoepithelial xenografts showed an increase in murine angiogenesis by both methods. This suggested that either the matrices of our myoepithelial xenografts or gene product(s) of the myoepithelial cells or both were inhibiting *neo*C8161-induced angiogenesis in vivo. We, in fact, found evidence of maspin, thrombospondin-1, TIMP-1, soluble bFGF receptors, prolactin and plasminogen fragments within 2M urea extracts of our myoepithelial xenografts. In tail vein injection studies of *neo*C8161, in mice harboring the myoepithelial xenografts, *neo*C8161 formed smaller pulmonary colonies than in mice harboring non-myoepithelial xenografts or in control mice (no xenografts) ($p<0.01$) (Fig. 2G, 2H, 2I). In a vWf factor immunocytochemical analysis of these smaller colonies in the mice harboring the myoepithelial xenografts, angiogenesis was minimal. These latter studies suggest the presence of circulating angiogenesis inhibitors released by the myoepithelial xenografts. Just recently we have demonstrated circulating maspin in mice harboring myoepithelial xenografts (see below).

Maspin and Myoepithelial Cells Can Be Manipulated Physiologically

Since PMA and dexamethasone were effective at pharmacologically altering maspin levels and the myoepithelial phenotype, we wondered whether physiological agents could do so as well. Because previous basic and clinical studies had examined the role of estrogen agonists and antagonists on human breast cancer cells and because issues of hormone replacement therapy (HRT) and tamoxifen chemoprevention are such timely issues in breast cancer, we wondered whether or not hormonal manipulations might affect myoepithelial cells in vitro as far as their paracrine suppressive activities on breast cancer were concerned. We recently demonstrated[12] that treatment of myoepithelial cells with tamoxifen but not 17-β estradiol increases both maspin secretion and invasion-blocking ability. 17-β Estradiol however competes with these suppressive effects of tamoxifen suggesting that the mechanism of tamoxifen action is estrogen receptor mediated. Myoepithelial cells lack ER-α but express ER-β. Tamoxifen, but not 17-β estradiol, increases AP-1 CAT but not ERE-CAT activity. Again, 17-β estradiol competes with the transcription-activating effects of tamoxifen. These experiments collectively suggest that the actions of tamoxifen on the increased secretion of maspin by myoepithelial cells may be mediated through ER-β and the trans-activation of an ER-dependent AP-1 response element.

As mentioned previously, immunoprecipitation of maspin from HMS-1 CM (Fig. 2F) reversed the anti-invasive effects of myoepithelial CM on breast carcinoma cell invasion in vitro. Tamoxifen treatment of HMS-1 resulted in a 2-3-fold increase in maspin secretion with increasing doses of tamoxifen and increasing times of exposure (Fig. 3A). 17-β Estradiol, in contrast, exerted no effects on maspin secretion and completely abolished the maspin stimulatory effects of tamoxifen in competition experiments. Tamoxifen's increase in maspin secretion was not due to an increase in steady state maspin mRNA levels which were essentially unchanged by this treatment. Myoepithelial cell lines lacked ER-α expression (Fig. 3B) but uniformly expressed ER-β (Figure 3B). Because the action of estrogen agonists/antagonists bound to estrogen receptors (either ER-α or ER-β) activates downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) or AP-1-tk-CAT. Tamoxifen (10^{-7} M) increased AP-1-CAT activity 3 fold (Fig. 3C). This effect was not observed with 17-β estradiol. Furthermore 17-β estradiol (10^{-5} M) competed with and blocked the effects of tamoxifen (10^{-7} M) (Fig. 3C). 17-β estradiol (10^{-7} M) did increase ERE-CAT activity but tamoxifen (10^{-7} M) did not.

Maspin and Myoepithelial Cells Are Potential Surrogate End Point Markers and Tumor Markers

Since maspin and myoepithelial cells seem intimately associated, and since myoepithelial cells are ubiquitous components of the ductal-lobular units of the breast and other organs which exhibit branching morphogenesis, we hypothesized that maspin might be detectable in fluid secreted by these ductal-lobular units. Since there has been a lot of recent interest in breast ductal fluid and breast nipple aspirates, especially, we measured maspin by Western blot and found it to be present in both nipple aspirates

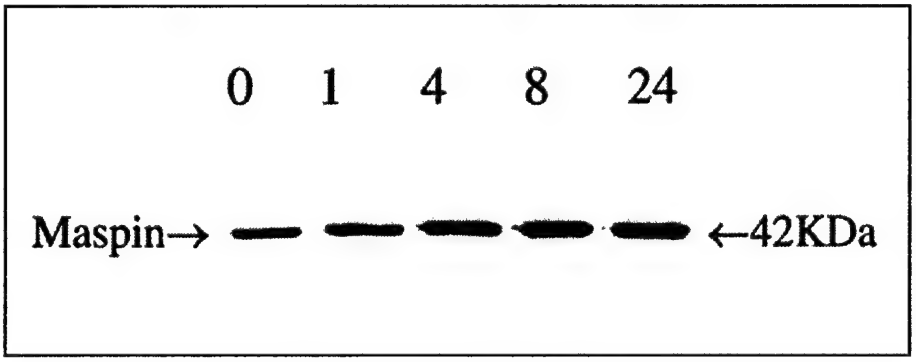

Fig. 3A. Effects of tamoxifen (10^{-7} M) treatment on maspin secretion for various times (hr) of tamoxifen exposure. 17β-Estradiol exerted no stimulatory effects. In competition experiments, increasing concentrations of 17β-estradiol completely blocked tamoxifen's stimulation of maspin secretion.

and ductal fluid (Fig. 4A) (Unpublished observations). This observation indicates that ductal fluid is not a mere transudate of blood or serum and that it is not a product of only epithelial cells (although epithelial protein products such as casein, lactalbumin and carcinoembryonic antigen (CEA) are certainly present). Ductal fluid also reflects a significant contribution from myoepithelial cells. From this observation we are currently studying groups of patients to see if their maspin levels serve to stratify them. We are currently analyzing ductal fluid collected following cannulation and washing of selected ducts in patients with microcalcifications on screening mammography who are about to undergo either excisional or core biopsy. Paired comparisons of maspin levels in ductal fluid obtained from ducts harboring microcalcifications or DCIS and normal ducts from the same patients are also being made. Maspin levels can be correlated with the histopathology surrounding the microcalcifications. It is anticipated that some of these patients will exhibit normal ductal histopathology surrounding their microcalcifications, some will harbor proliferations like hyperplasia, adenosis, ADH, and DCIS and still others invasive carcinoma. The screening value of maspin levels in all of these patients can be determined. Measurements of myoepithelial maspin in ductal fluid will be compared to levels of a breast epithelial cell marker such as CEA. CEA has been observed to be increased in nipple secretions and ductal fluid in patients with ductal hyperplasia. Hence the maspin/CEA ratio might be predictive of risk with increased maspin/CEA correlating with normalcy and decreased maspin/CEA correlating with either high risk, microcalcifications and/or precancerous histopathology. In this sense maspin can be used as a surrogate end point marker to predict either the risk of DCIS or the likelihood that DCIS will progress to invasive breast cancer.

Another interesting observation with respect to the use of myoepithelial maspin as a marker, this time, a tumor marker, is the observation that maspin can be detected in normal saliva but that it is markedly elevated in saliva secreted from a salivary gland neoplasm and that it is also elevated in murine serum in mice harboring human myoepithelial xenografts (Fig.4B) (unpublished observations). Most salivary gland neoplasms are thought to be myoepithelial in origin. These include mixed tumors, basal

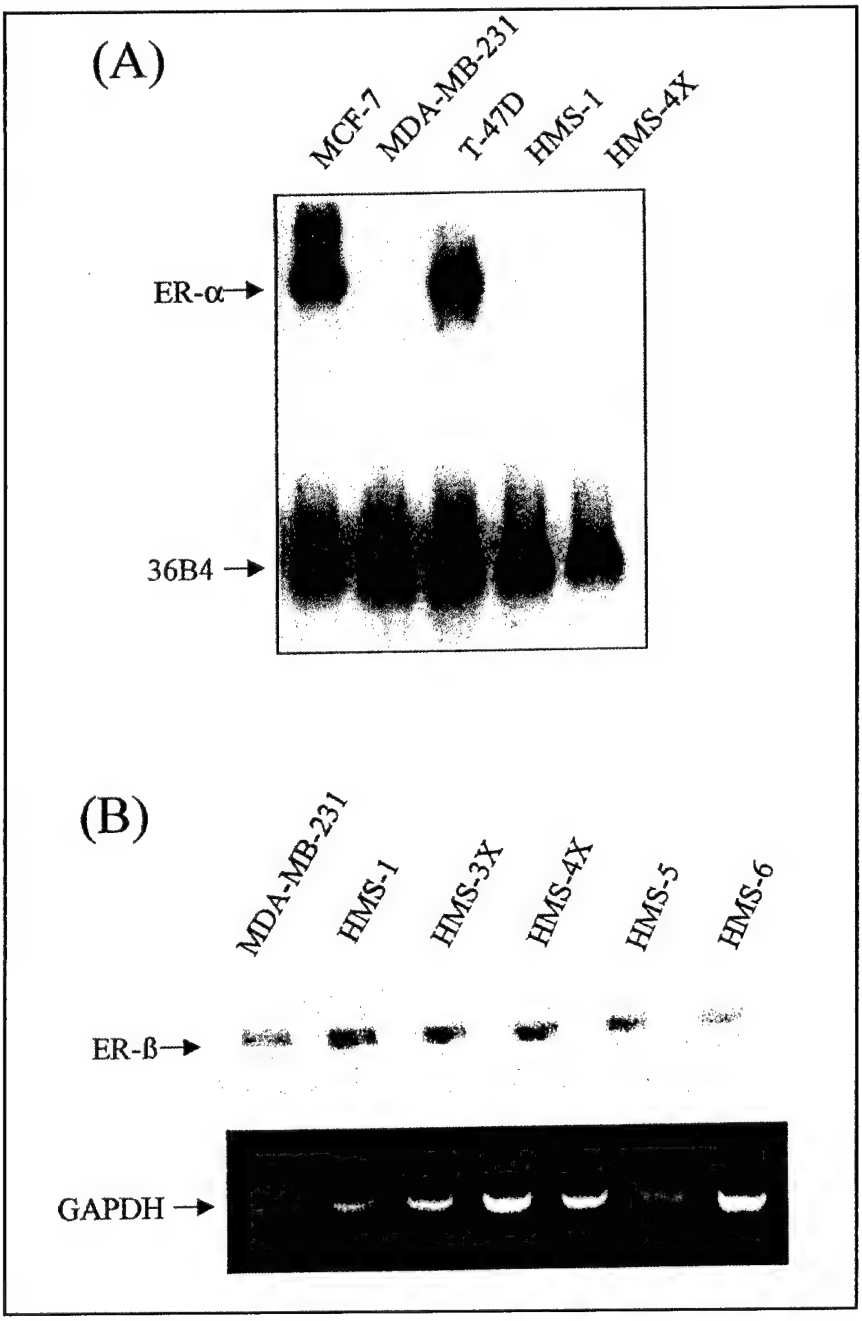

Fig. 3B. Expression of ER-α and ER-β in representative myoepithelial cell lines. [A] Northern blot analysis, ER-α expression. Normalization was with 36B4. [B] ER-β expression by RT-PCR. GAPDH served as a housekeeping control.

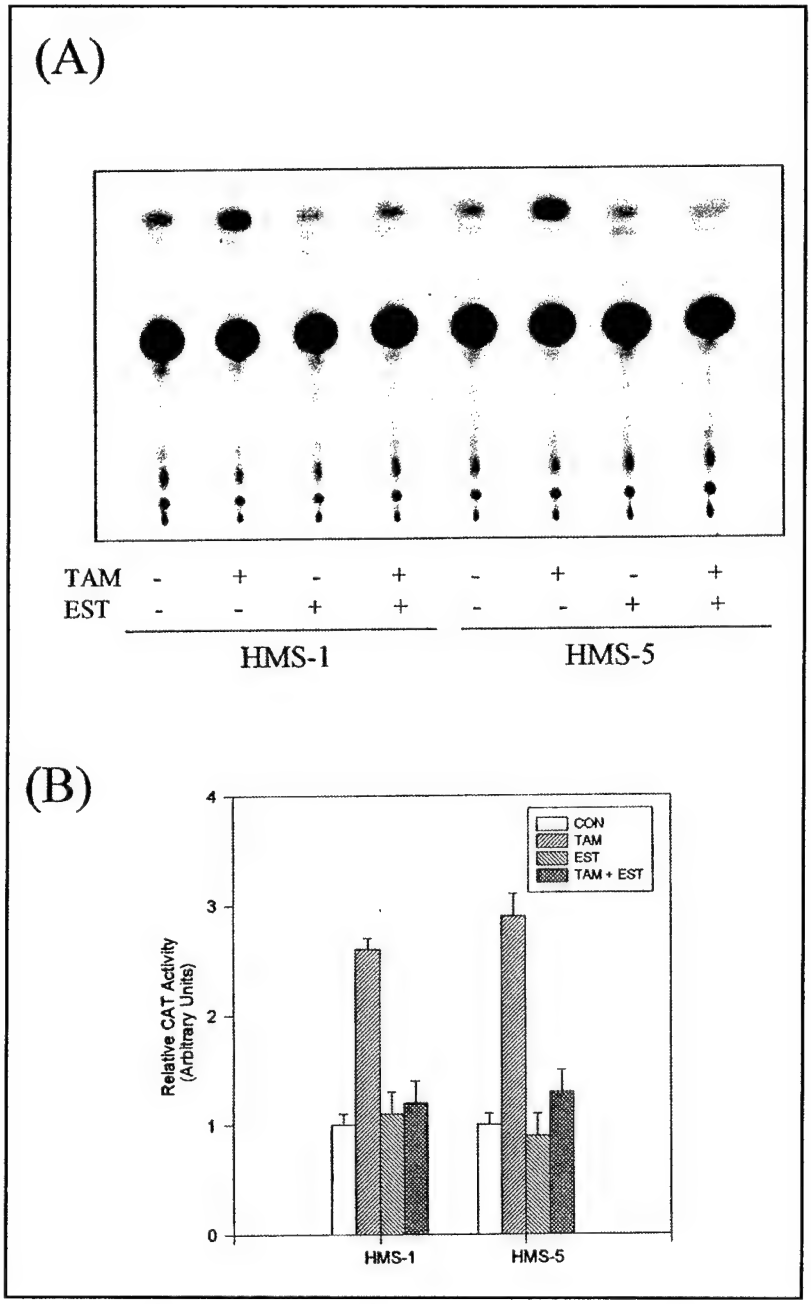

Fig. 3C. Tamoxifen (10^{-7} M) stimulation of AP-1-CAT activity in representative myoepithelial cell lines, HMS-1 and HMS-5 [A]. 17-β Estradiol (10^{-7} M) exerted no such stimulatory effects and blocked the effects of tamoxifen at high doses (10^{-5} M). [B] Results depicted are the means of three independent experiments. Error bars represent standard errors

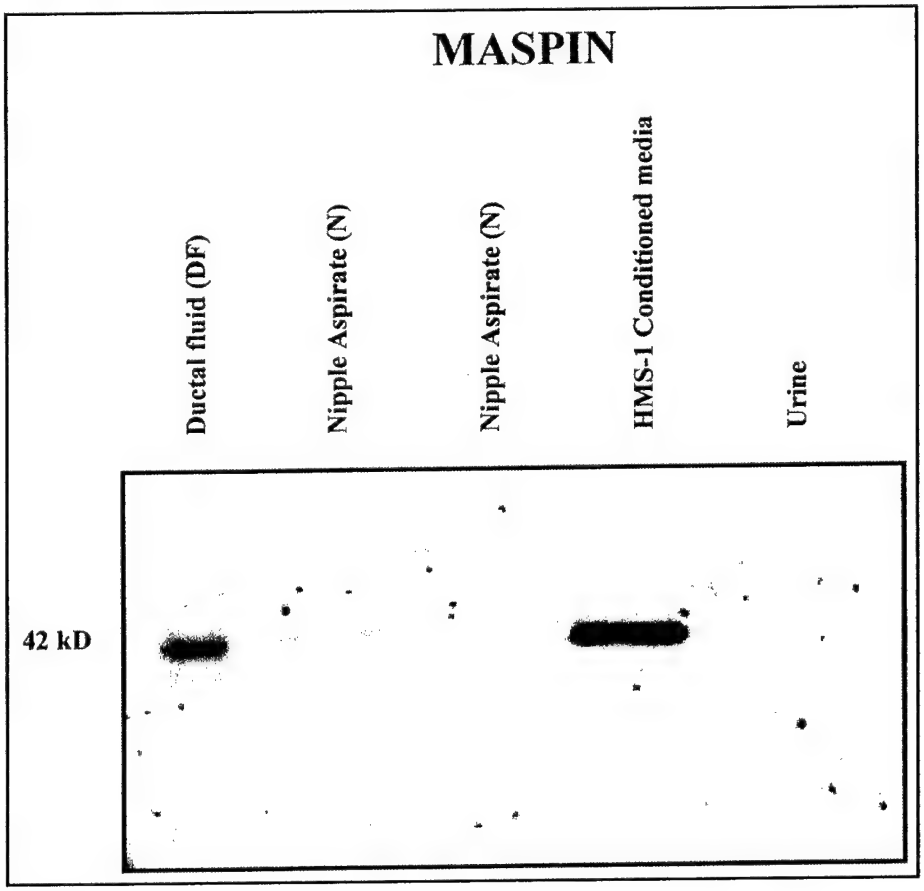

Fig. 4A. Nipple aspirate fluid (N) collected by nipple suction or ductal fluid (DF) collected by selected ductal cannulation can be studied for protein composition. In the former method (nipple aspiration), the contributions of each of the ductal systems can not be distinguished from each other; however in the latter method (ductal cannulation), each of the ductal systems can be separately analyzed and therefore ductal fluid from ducts harboring DCIS, for example, can be compared with ductal fluid from normal ducts in the same patient. Both nipple aspirate fluid and ductal fluid reflect the secretory contributions of the ductal-lobular units which are composed of both myoepithelial cells as well as epithelial cells. Maspin, a secretory product of myoepithelial cells, can be detected as a 42 kDa protein on Western blot from both nipple aspirate fluid (N) as well as ductal fluid (DF). Conditioned media (CM) from HMS-1, a myoepithelial cell line, is also intensely positive for maspin. Urine is negative.

cell adenomas, basal cell adenocarcinomas, and adenoid cystic carcinomas. It was human tumors of these types that originally gave rise to our myoepithelial cell lines/xenografts that led to a dissection of the myoepithelial phenotype and to our observations concerning myoepithelial maspin. If screening saliva for maspin shows promise for detecting small incipient salivary gland neoplasms, then myoepithelial maspin will show its utility as a tumor marker. So, in summary, our findings indicate that maspin and myoepithelial cells contribute to the structural and functional integrity of the ductal-lobular units of different organs, and alterations in maspin levels in fluid from these units may reflect disease states (Fig.4C).

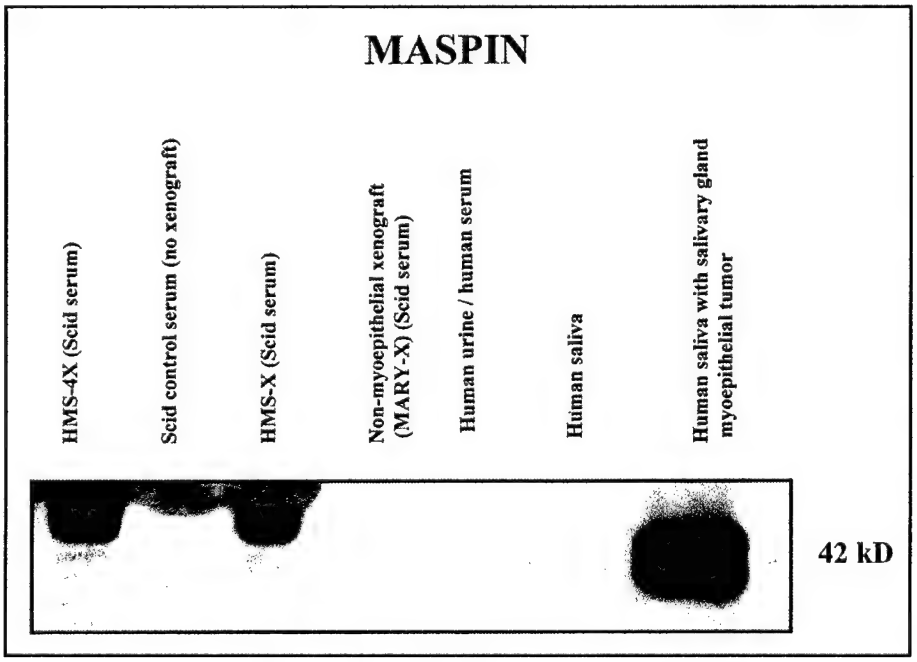

Fig. 4B. The contribution of myoepithelial tumors to maspin secretion in bodily fluids is demonstrated. In the Scid mouse, mice with >1 cm human myoepithelial xenografts (HMS-X, HMS-4X) exhibit circulating maspin levels in serum. Mice harboring no xenograft or a non-myoepithelial xenograft (MARY-X, an inflammatory breast carcinoma xenograft) contain no detectable circulating maspin. Human saliva in normal patients contain low levels of maspin, presumably from the myoepithelial cells in the salivary gland ductal-lobular units. Saliva from patients with benign salivary gland myoepithelial tumors show markedly elevated maspin.

Future Directions

The observations that myoepithelial cells secrete maspin in large quantities whereas carcinoma cells do not suggest that maspin and myoepithelial cells exert pleiotropic suppressive effects on tumor progression. Since maspin is both a proteinase inhibitor, a locomotion inhibitor and an angiogenesis inhibitor, the diverse actions of maspin may largely explain the pronounced anti-invasive and anti-angiogenic effects of myoepithelial cells on carcinoma and pre-carcinoma cells (13-24). Clearly, maspin and myoepithelial cells have more than marker value. Circulating maspin may have value as an anti-angiogenic agent. We need to better understand what it is about the myoepithelial phenotype that allows for high constitutive expression and secretion of maspin. Studies of the maspin promoter and *cis/trans* interactions within the myoepithelial cell seem to be an attractive line of further research. We also need to better understand the mechanism by which certain pharmacological agents such as PMA and certain physiological agents such as tamoxifen bolster myoepithelial secretion of maspin. With this understanding we may be able to design small less toxic molecules that have the same effect. We need to better exploit the intricate paracrine and local relationships which exist between myoepithelial cells and epithelial cells (precancerous and cancerous) in the breast and other organs. This is especially important and timely as

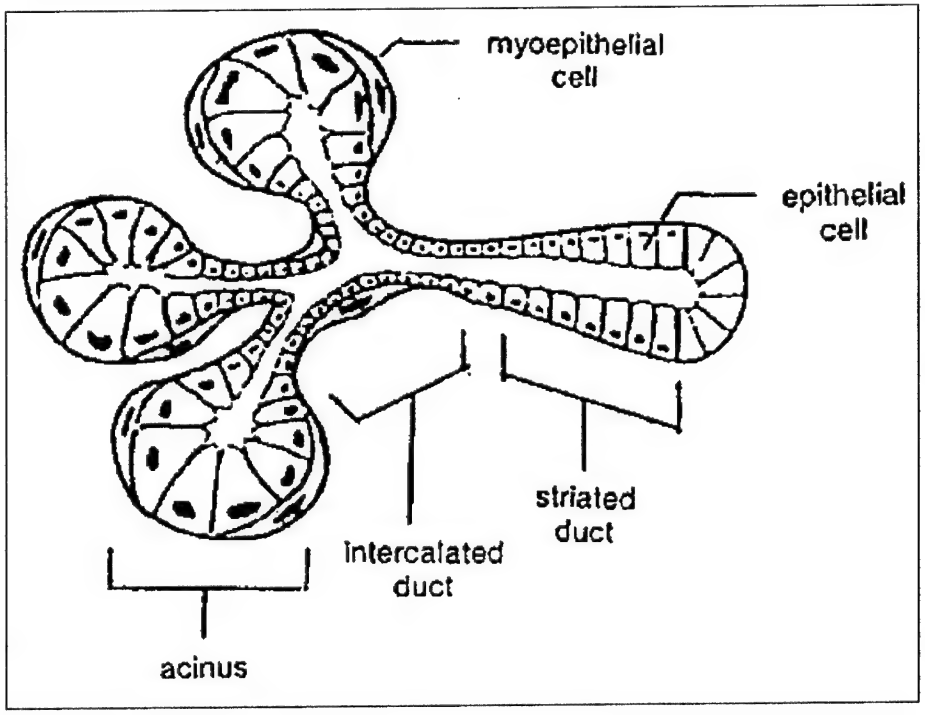

Fig. 4C. Schematic depicts the ductal-lobular unit of the breast and/or salivary gland and emphasizes the point that both myoepithelial cells as well as epithelial cells contribute to the integrity of this unit and to the composition of ductal fluid and saliva. In the case of invasive breast cancer, the integrity of the myoepithelial layer may be compromised and maspin levels may decrease. In the case of a myoepithelial tumor of salivary glands, the secretion of maspin into saliva may serve as a tumor marker.

intraductal approaches through the nipple are gaining in popularity as a means of screening women who are at risk for developing breast cancer. These intraductal approaches really exploit the local myoepithelial/epithelial relationships which exist. Screening for maspin levels as a surrogate end point marker is only the beginning. One could envision delivering intraductal gene therapy designed to exploit the inherent differences between myoepithelial and epithelial cells. One could target and destroy the epithelial cells selectively sparing the myoepithelium or alternately target the myoepithelial cells with a vector which bolsters its secretion of maspin. If the myoepithelial defense can be bolstered in this manner, perhaps this natural barrier which normally inhibits invasion for years can be made into an impervious barrier which inhibits invasion forever. At least that is one vision of scientists who are interested in maspin and myoepithelial cells.

Abbreviations

CM, conditioned medium; FCS, fetal calf serum; DCIS, ductal carcinoma in situ; PN-II/APP, protease nexin II/β-amyloid precursor protein; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase-type

plasminogen activator; PAI, plasminogen activator inhibitor; α1-AT, α1-antitrypsin; HMEC, human mammary epithelial cells; K-SFM, keratinocyte serum-free medium; vWf, von Willebrand factor; CHX, cyclohexamide; dB-cAMP, N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate; Na-But, sodium butyrate; RA, all *trans* retinoic acid; 5-azaC, 5-azacytidine; PMA, phorbol 12-myristate 13-acetate: UVE, umbilical vein endothelial cells; bFGF, basic fibroblast growth factor.

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10

Myoepithelium: Methods of Culture and Study

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1. INTRODUCTORY REVIEW

Paracrine regulation of tumor progression by host cells is an important determinant of tumor growth, invasion, and metastasis. However, one cell that has largely been ignored in this regulation is the myoepithelial cell. In any organ where there is significant branching morphogenesis, such as the breast, myoepithelial cells ubiquitously accompany and surround epithelial cells and are thought to keep in check (negatively regulate) the process of branching. Myoepithelial cells surround both normal ducts and precancerous lesions, especially of the breast (so-called DCIS, ductal carcinoma in situ), and form a natural border separating proliferating epithelial cells from proliferating endothelial cells (angiogenesis). Myoepithelial cells, by forming this natural border, are thought to negatively regulate tumor invasion and metastasis. Whereas epithelial cells are susceptible targets for transforming events leading to cancer, myoepithelial cells are resistant. Indeed tumors of myoepithelial cells are uncommon and, when they do occur, are almost always benign. Therefore, it can be said that myoepithelial cells function as both autocrine as well as paracrine tumor suppressors.

Our laboratory has found that myoepithelial cells secrete numerous suppressor molecules: high amounts of diverse proteinase inhibitors, which include maspin, TIMP-1, protease nexin-II, and α-1 antitrypsin; low amounts of proteinases and high amounts of diverse angiogenic inhibitors, which include maspin, thrombospondin-1, and soluble bFGF receptors, but low amounts of angiogenic factors compared with common malignant cell lines. Whereas carcinoma cells secrete more proteinases than proteinase inhibitors and more angiogenic factors than angiogenic inhibitors, myoepithelial cells then do just the opposite. This observation holds in vitro, in mice and in humans, and suggests

that myoepithelial cells exert pleiotropic suppressive effects on tumor progression.

This constitutive gene expression profile of myoepithelial cells may largely explain the pronounced anti-invasive and anti-angiogenic effects of myoepithelial cells on carcinoma and pre-carcinoma cells and may also account for the low-grade biology of myoepithelial tumors, which are devoid of appreciable angiogenesis and invasive behavior. Many of the secretory gene products of myoepithelial cells are present in body fluids, such as in breast ductal fluid and in saliva, reflecting the structural and functional integrity of the ductal-lobular units of the mammary and salivary glands, respectively. Some of the myoepithelial gene products; for example, maspin, in ductal fluid may serve as a surrogate (intermediate) end-point marker (SEM) to estimate the risk of DCIS progression to invasive cancer in the breast and, alternatively, in saliva, may serve as a tumor marker to detect the presence of incipient myoepithelial tumors occurring within the salivary glands of the head and neck.

In order to study the cell and molecular biology of myoepithelial cells further, methods to isolate and characterize human myoepithelial cells must be devised and perfected. Two sources of human myoepithelial cells are 1) the normal ductal-lobular units of organs rich in myoepithelial cells, such as the breast and salivary glands; and 2) benign tumors of myoepithelial cells where large numbers of myoepithelial cells, albeit transformed, can be obtained and used as normal myoepithelial cell surrogates.

2. STUDIES OF MYOEPITHELIAL CELLS

It has become clear that cancer cells come under the influence of important paracrine regulation from the host microenvironment [Cavenee, 1993]. Such host regulation may be as great a determinant of tumor cell behavior in vivo as the specific oncogenic or tumor suppressor alterations occurring within the malignant cells themselves, and may be mediated by specific extracellular matrix molecules, matrix-associated growth factors, or host cells themselves [Liotta et al., 1991; Safarians et al., 1996]. Both positive (fibroblast, myofibroblast, and endothelial cell) and negative (tumor-infiltrating lymphocyte and cytotoxic macrophage) cellular regulators exist, which profoundly affect tumor cell behavior in vivo [Folkman and Klagsbrun, 1987; Cornil et al., 1991]. However, one host cell, the myoepithelial cell, has escaped the paracrine onlooker's attention. The myoepithelial cell, which lies on the epithelial side of the basement membrane, is thought to contribute largely to both the synthesis and remodeling of this structure. This cell lies in juxtaposition to normally proliferating and

differentiating epithelial cells in health and to abnormally proliferating and differentiating epithelial cells in precancerous disease states, such as DCIS of the breast. This anatomical relationship suggests that myoepithelial cells may exert important paracrine effects on normal glandular epithelium and may regulate the progression of DCIS to invasive breast cancer. Circumstantial evidence suggests that the myoepithelial cell naturally exhibits a tumor suppressive phenotype. Myoepithelial cells rarely transform, and, when they do, they generally give rise to benign neoplasms that accumulate rather than degrade extracellular matrix [Guelstein et al., 1993]. Myoepithelial cells directly or indirectly through their production of extracellular matrix and proteinase inhibitors, including maspin, are thought to regulate branching morphogenesis that occurs in the developing breast and salivary gland during embryological development [Cutler, 1990]. There have been a paucity of studies on myoepithelial cells because they have been relatively difficult to culture and because tumors that arise from these cells are rare.

In previous studies we have been extremely fortunate to have successfully established immortalized cell lines and transplantable xenografts from benign or low-grade human myoepitheliomas of the salivary gland and breast [Sternlicht et al., 1996; Sternlicht et al., 1997]. These lines/xenografts have been designated HMS-#; HMS-#X, respectively. These pneumonics stand for human matrix secreting line and xenograft, respectively and refer to the chronological order of establishment. To date six lines/xenografts numbered HMS-1–6 and HMS-X–6X have been established. These lines and xenografts are available to investigators on request. These cell lines and xenografts displayed an essentially normal diploid karyotype and expressed identical myoepithelial markers as their in situ counterparts, including high constitutive expression of maspin. Unlike the vast majority of human tumor cell lines and xenografts, which exhibited matrix-degrading properties, these myoepithelial lines/xenografts, like their myoepithelial counterparts in situ, retained the ability to secrete and accumulate an abundant extracellular matrix composed of both basement membrane and non-basement membrane components. When grown as a monolayer, one prototype myoepithelial cell line, HMS-1, exerted profound and specific effects on normal epithelial and primary carcinoma morphogenesis [Sternlicht et al., 1996]. These studies support our position that our established myoepithelial lines/xenografts recapitulate a normal differentiated myoepithelial phenotype and can therefore be used experimentally as a primary myoepithelial cell surrogate. Prompted by these studies and by the conspicuous absence of studies examining the role of the myoepithelial cell in tumor progression, we decided to examine the myoepithelial cell from this perspec-

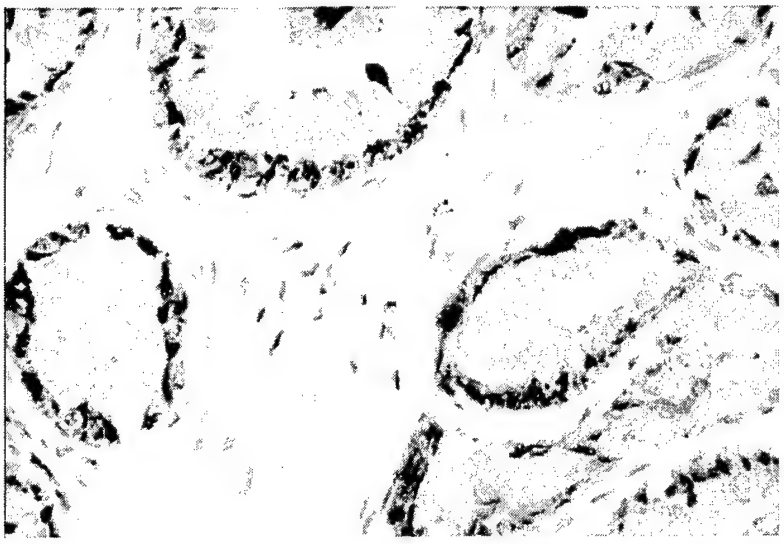
tive. Experiments with these cell lines/xenografts together with relevant in situ observations form the cornerstone of our studies, which observe that the human myoepithelial cell is a natural tumor suppressor.

3. INHIBITION OF TUMOR INVASION

Breast ducts and acini are surrounded by a circumferential layer of myoepithelial cells exhibiting strong immunoreactivity for S100, smooth-muscle actin, CALLA, calponin, and diverse proteinase inhibitors, including maspin, α1-AT, PNII/APP, and TIMP-1 (Fig. 10.1A). In DCIS, the myoepithelial layer appeared either intact or focally disrupted, but the myoepithelial cells themselves exhibited the same pattern of immunoreactivity (Fig. 10.1B). In DCIS, although proliferations of vWf immunoreactive blood vessel capillaries were observed focally within the supporting stroma, such blood vessels were not observed within the proliferating DCIS cells on the epithelial side of the myoepithelial layer (Fig. 10.1C). The human tumoral-nude mouse xenografts derived from the human myoepitheliomas of the salivary gland, HMS-X and HMS-3X, and breast, HMS-4X demonstrated immunocytochemical profiles identical to each other and to that exhibited by the myoepithelial cells surrounding normal ducts and DCIS with especially intense maspin immunoreactivity (Fig. 10.1D).

Not only was strong proteinase inhibitor immunoreactivity present within the myoepithelial cells of these xenografts, but strong proteinase inhibitor immunoreactivity could be demonstrated within their extracellular matrix as well. Due to this matrix, the myoepithelial xenografts appeared white and cartilaginous in nature (Fig. 10.1E). Within this abundant extracellular matrix deposited by the different human myoepithelial xenografts, murine blood vessels were not observed (Fig. 10.1F). Through the use of a mouse-specific Cot-1 DNA probe (Fig. 10.1G), human myoepithelial xenografts HMS-X, HMS-3X, and HMS-4X demonstrated absent or near-absent angiogenesis. Human non-myoepithelial xenografts of breast cancer cell lines MDA-MB-231 and MDA-MB-468, in contrast, showed a comparatively large murine component of angiogenesis. In a two-dimensional matrix, myoepithelial cell lines grew as a confluent monolayer with self-forming spheroids at superconfluency (Fig. 10.1H). In a three-dimensional matrix, myoepithelial cell lines branched and budded (Fig. 10.1I). In monolayer culture, ultrastructural studies confirmed the cells' myoepithelial identity (Fig. 10.1J).

Detailed studies [Sternlicht et al., 1997] conducted with HMS-1, a prototype myoepithelial cell line, revealed a constitutively high proteinase inhibitor to proteinase ratio in strong contrast to the high



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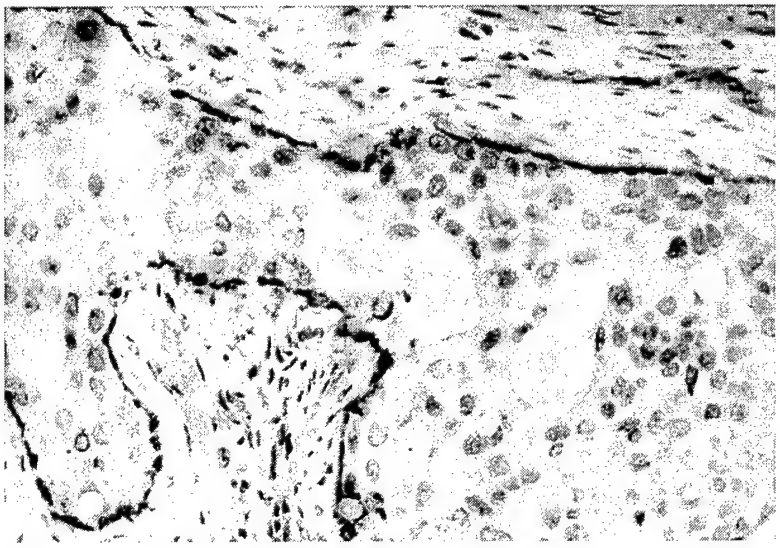
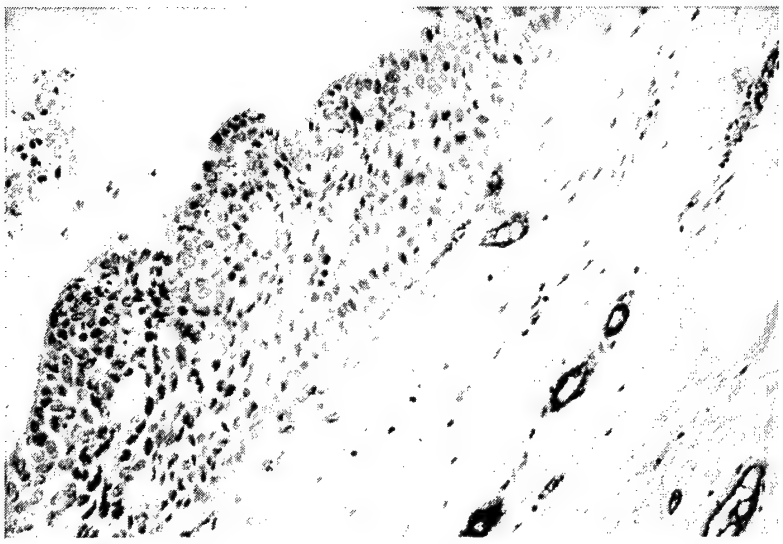


B

Figure 10.1. *In situ* immunocytochemistry profile of myoepithelial cells and their derived cell lines/xenografts. A) Differential maspin immunoreactivity of myoepithelial cells surrounding breast ducts and acini. B) Differential maspin immunoreactivity of myoepithelial cells in DCIS. C) Angiogenesis demonstrated by vWf immunoreactivity limited to stromal side of DCIS. D) Cytoplasmic maspin immunoreactivity of myoepithelial xenograft, HMS-X. E) Gross appearance of one myoepithelial xenograft, HMS-X. F) Microscopic appearance of one typical myoepithelial xenograft, HMS-4X, devoid of apparent angiogenesis. G) Murine Cot-1 dot blot. With a mouse-specific Cot-1 DNA probe, human myoepithelial xenografts, HMS-X, HMS-3X,



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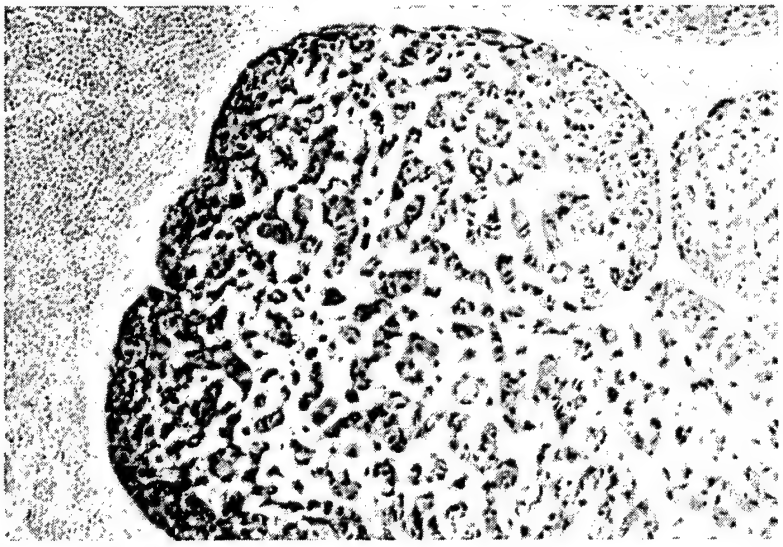
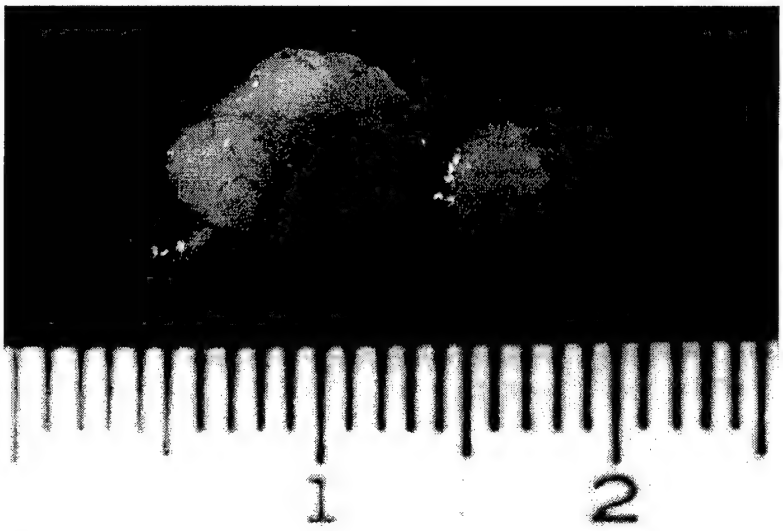


D

Figure 10.1. *(Continued)*
and HMS-4X are devoid of a murine DNA angiogenic component in contrast to the angiogenic-rich MDA-MB-231-X and MDA-MB-468-X breast carcinoma xenografts (right column); control dot blots of varying murine DNA percentages are also depicted (left column). H) The prototype myoepithelial cell line, HMS-1, in culture, grows as self-inducing spheroids on top of its own monolayer. I) HMS-1 undergoes branching morphogenesis and budding when grown on either Matrigel or a myoepithelial-derived matrix, *Humatrix*. J) Myoepithelial cells in culture, ultrastructurally, give the impression of "smiling," which is symbolic of the cells' natural tumor-suppressive function.



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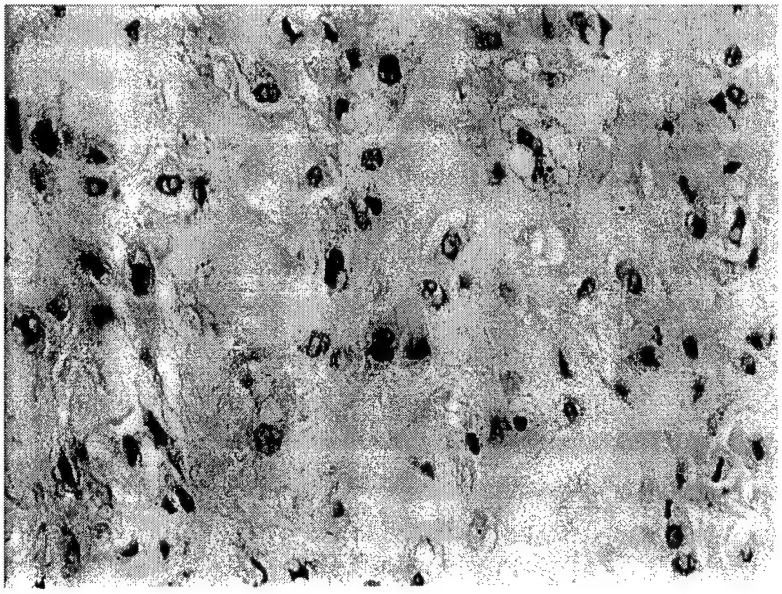
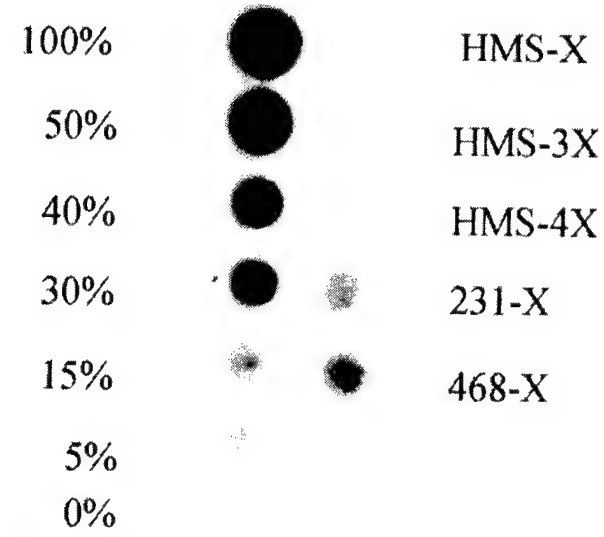


F

Figure 10.1. *(Continued)*



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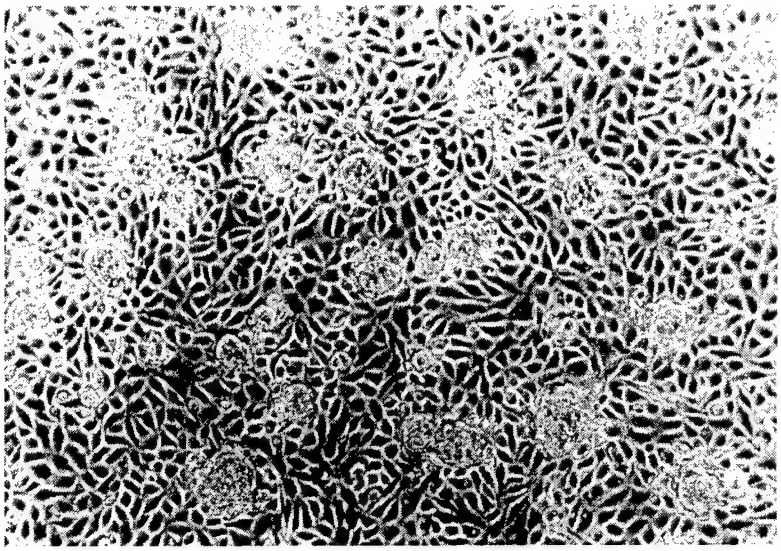

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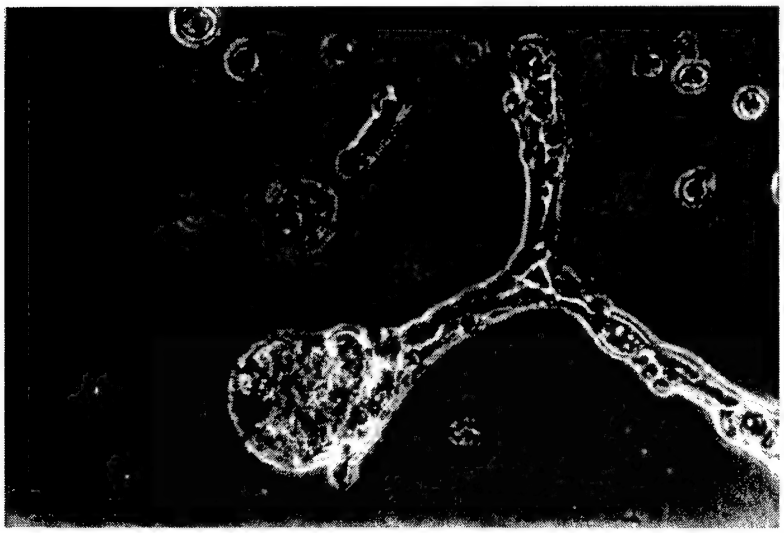

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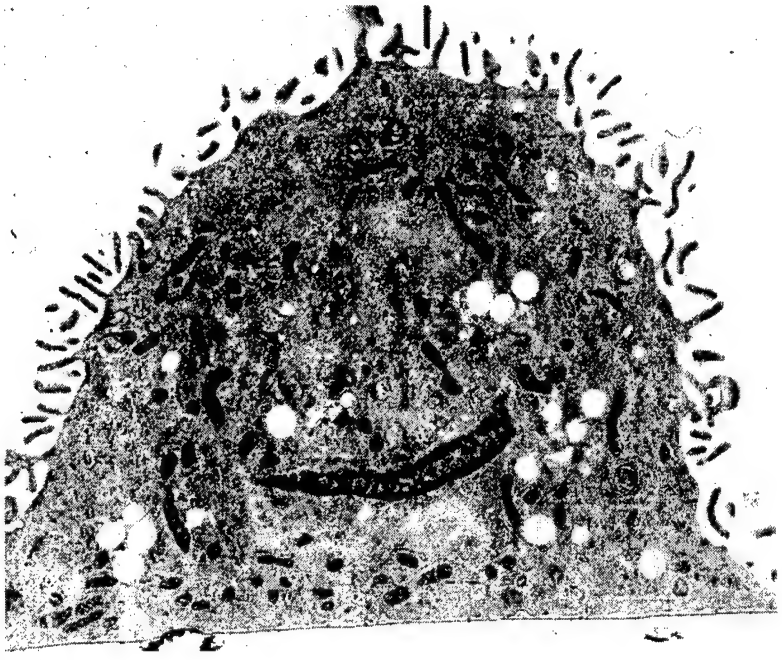


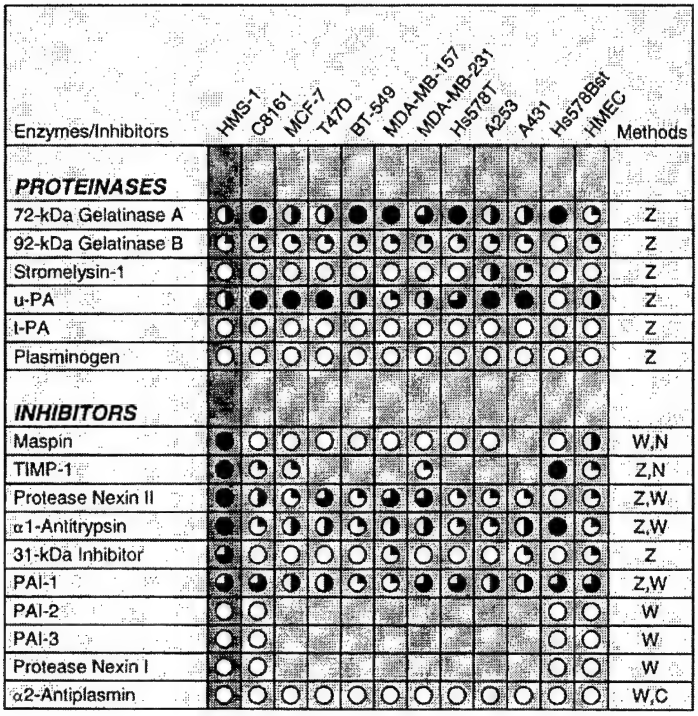
J

Figure 10.1. *(Continued)*

proteinase to proteinase inhibitor ratio observed in a number of malignant human cell lines (Fig. 10.2A). Marker studies with this cell line and corresponding xenograft (HMS-X) reflected the constitutive gene expression profile of myoepithelial cells in situ (Fig. 10.2B). This finding was especially true with respect to maspin. Direct gelatin zymography of conditioned media (CM) revealed only low levels of the 92 and 72 kDa type IV collagenases (MMP-9 and MMP-2, respectively) in HMS-1; the 72 kDa collagenase was reduced sixfold in HMS-1 compared with the levels in the majority of the malignant lines; direct fibrin zymography revealed visibly lower levels of the 54 kDa urokinase plasminogen activator (uPA) in HMS-1. This condition was also observed in casein/plasminogen gels.

Tissue-type plasminogen activator was not detected in any cell line, nor was plasmin detected in control gels lacking plasminogen. Stromelysin-1 (MMP-3) was also not detected in HMS-1. The proteinase inhibitor expression profile of HMS-1, in contrast, was characterized by high constitutive expression in CM of several proteinase inhibitors, including TIMP-1; PAI-1; three trypsin inhibitors: α1-AT, PNII/APP, and an unidentified 31 kDa inhibitor detected initially on reverse zymography; and the tumor suppressor maspin. With respect to the trypsin serine proteinase inhibitors, the conspicuous doublet at 116 kDa consistently greater in HMS-1 than in any of the other lines examined was confirmed on Western blot as PNII/APP. These bands represented the 770 and 751 amino acid isoforms of PNII/APP, which possessed a Kunitz-type serine proteinase inhibitor domain.

Interestingly, in 2 M urea extracts of HMS-X, HMS-3X, and HMS-4X, a novel 95 kDa band of trypsin inhibition was detected by reverse-zymography and was confirmed by Western blot to represent an active breakdown product of PNII. This 95 kDa PNII breakdown product was completely absent from HMS-1 CM and urea extracts of HMS-1 cells, suggesting that it was produced in situ within the myoepithelial extracellular matrix to which it bound. The retention of proteinase inhibitor activity by this breakdown product indicated that it retained the Kunitz-type serine proteinase inhibitor domain responsible for its ability to inhibit trypsin. In contrast to PNII/APP, protease nexin I was not detected. The second trypsin serine proteinase inhibitor was present at 54 kDa and was α1-AT. This inhibitor appeared nearly equivalent in HMS-1 compared with the malignant lines examined on reverse-zymography, but by Western blot its signal was markedly stronger and slightly more mobile in HMS-1 than in the malignant lines. These data were reconciled with the fact that α1-AT was probably less glycosylated in HMS-1. This relative underglycosylation caused α1-AT from HMS-1 to migrate slightly further into the gel and accounted for its poorer reactivation following sodium dodecyl sulfate

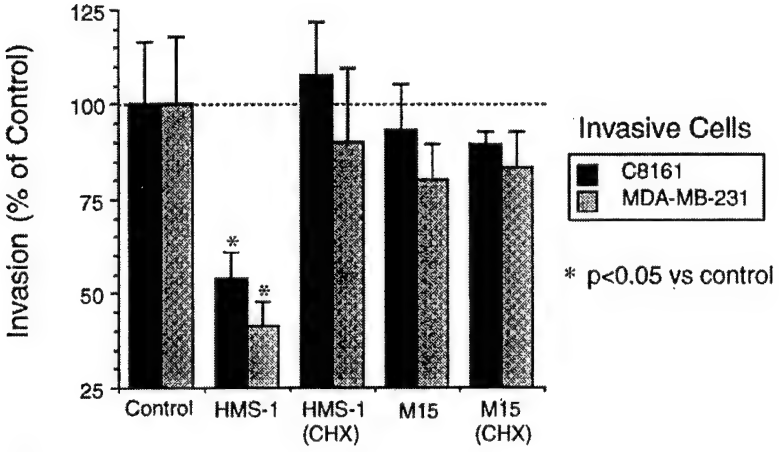


A

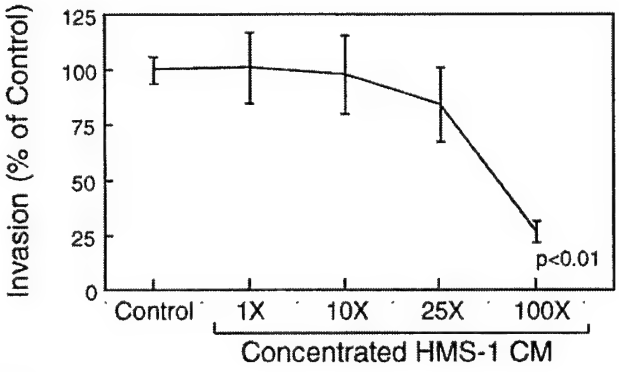
	HMS-X,3X,4X		Normal Breast		DCIS	
	Cells	Matrix	ME†	Epi†	ME	Epi
S-100	++++*	-	++++	-	++++	-
Maspin	++++	-	++++	+	++++	±
α1-AT	++	++	++	-	++	-
PNII	++	+++	++	-	++	±
TIMP-1	++	+	++	-	++	-
PAI-1	+	±	+	+	+	+
vWf	-	-	-§	-§	-§	-§

B

Figure 10.2. A) Relative constitutive expression of diverse proteinase inhibitors and proteinases in myoepithelial cells (HMS-1) compared with various malignant cell lines. Z, direct or reverse zymography; W, Western blot; N, Northern blot; C, chromogenic substrate assay. B)

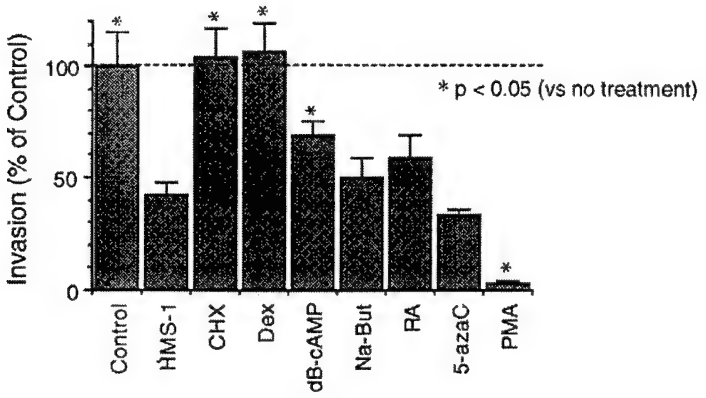


C



D

Figure 10.2. *(Continued)*
Myoepithelial-related immunoreactivity *In Situ.* [†]myoepithelial cells; [‡]epithelial cells; *++++, intensely positive; +++, strongly positive; ++, positive; +, weakly positive; ±, equivocally positive; −, negative; [§]on epithelial side of basement membrane. C) Effects of HMS-1 cells on C8161 (melanoma) and MDA-MB-231 (breast carcinoma) invasion. D) Effects of HMS-1 CM on invasion of C8161 cells. Results with MDA-MB-231 cells were similar. Assays in both (C, D) were performed in quadruplicate and show mean percentage of control invasion ± standard deviation. E) Effects of pharmacological treatment of HMS-1 cells with various agents inducing invasion-permissive and non-permissive phenotypes: CHX, cycloheximide; DEX, dexamethasone; dB-cAMP, N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; Na-But, sodium butyrate; RA, all *trans* retinoic acid; 5-azaC, 5-azacytidine; PMA, phorbol 12-myristate 13-acetate. Dexamethasone induced invasive-permissive phenotype, whereas PMA induced a non-permissive phenotype. Invasion (percentage of control) of C8161 melanoma cells is depicted.



E

Figure 10.2. *(Continued)*

(SDS)-denaturation on reverse-zymography compared with the more highly glycosylated isoforms present in the malignant lines.

The third trypsin serine proteinase inhibitor detected at 31 kDa was clearly not a degradation product of either PNII or α1-AT, as demonstrated by negative Western blot. The 31 kDa inhibitor was strongly expressed in HMS-1 and was either absent or nearly absent in all of the malignant lines examined. It is being determined whether this unidentified inhibitor is a novel inhibitor. In contrast to the above inhibitors, PAI-1 was expressed only slightly greater in HMS-1 compared with the majority of the malignant lines by both reverse-zymographic and Western blot analysis. Neither PAI-2, PAI-3 or α2-antiplasmin were detected by Western blot analysis in any of the cell lines. Antiplasmin activity, as determined by photometric assay, was completely absent as well.

The most striking difference, however, between the strong proteinase inhibitor profile of HMS-1 and the profile of the malignant cell lines examined was in the expression of maspin. Intense maspin transcripts (3.0 and 1.6 kb) and protein (42 kDa) were identified in HMS-1 and HMS-1 CM, respectively, but were completely absent in all of the malignant lines examined (Fig. 10.2A). With its proteinase inhibitor profile of increased maspin, TIMP-1, PN-II, α1-AT, and the 31 kDa inhibitor, HMS-1 bore strong resemblance to normal human mammary epithelial cells (HMEC, Clonetics) (Fig. 10.2A) except that the expression of all of these proteinase inhibitors, including maspin, was even more enhanced in HMS-1. Being derived from normal ducts and acini of the human breast, HMEC cultures likely contain myoepithelial as well as epithelial cells. Thus, the resemblance of HMS-1 to

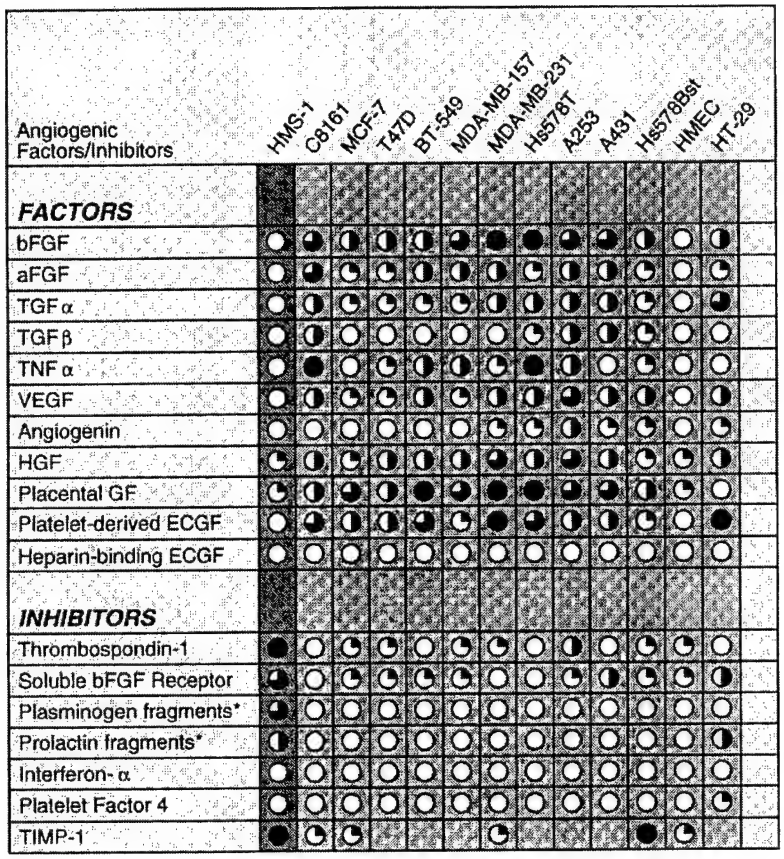
HMEC further supported our contention that HMS-1, though immortal, expressed a well-differentiated myoepithelial phenotype. In addition, because HMS-1 was a clonal line expressing a pure myoepithelial phenotype, it would be predicted to express certain myoepithelial-associated proteins, such as maspin, α1-AT, PNII/APP, and TIMP-1, to a greater degree than HMEC. Predictably, the myofibroblast line Hs578Bst was strongly expressive of TIMP-1 but did not express maspin, PNII, or the 31 kDa inhibitor (Fig. 10.2A). The strong proteinase inhibitor profile exhibited by HMS-1 was shared by all of the myoepithelial xenografts, including HMS-X, HMS-3X, and HMS-4X.

In the modified Matrigel invasion chamber used in this study, HMS-1 cells and their CM dramatically inhibited invasion of two invasive melanoma and breast carcinoma cell lines (Fig. 10.2C, D). The HMS-1 line was itself non-invasive in this chamber. Predictably, the anti-invasive effects of HMS-1 could be abolished by CHX (40 μg/ml) 24-h pretreatment. HMS-1 CM inhibited invasion in a dose-response fashion up to 30% ± 8% of control ($P < 0.01$) (Fig. 10.2D). Pretreatment of HMS-1 with dexamethasone (0.25 μM) produced a complete invasion-permissive phenotype (100% of control), whereas pretreatment with phorbol 12-myristate 13-acetate (PMA; 5 μM) produced an essentially nonpermissive phenotype (2% of control) ($P < 0.05$) (Fig. 10.2E). The effects of dexamethasone and PMA were quite dramatic. The effects of other agents, including RA, dB-cAMP, Na-But, and 5-azaC, showed either permissive or non-permissive trends but were less dramatic. PMA's induction of the nonpermissive phenotype began after 20 min pretreatment, was almost complete after 2 h, and was maximized after 24 h ($P < 0.05$). The induction of this nonpermissive phenotype correlated with the induction of a dramatic fivefold increase in maspin secretion measured in HMS-1 CM. As a result of PMA treatment, both an immediate release (within 2 min) of maspin from HMS-1 cells occurred, as well as a more sustained secretion for at least 24 h following PMA pretreatment. The increased maspin secretion was not on the basis of an increase in steady-state maspin transcripts. PMA also resulted in a less dramatic twofold increase in both MMP-9 and TIMP-1 secretion. Dexamethasone's induction of an invasion-permissive phenotype in HMS-1 was not associated with a change in either maspin transcription or secretion. Immunoprecipitation with anti-maspin antibody at 1/100 dilution successfully removed all detectable maspin from myoepithelial cell CM. This CM lost its ability to inhibit invasion. Similar results were observed with the CM from the other myoepithelial lines (HMS-3 and HMS-4) studied. None of the non-myoepithelial cell CM inhibited invasion.

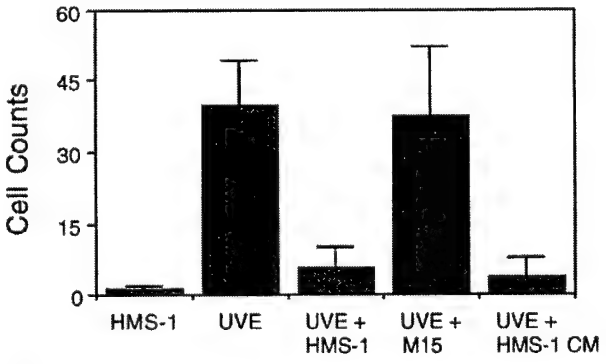
4. INHIBITION OF TUMOR ANGIOGENESIS

Human myoepithelial cells, which surround ducts and acini of certain organs such as the breast, form a natural border separating epithelial cells from stromal angiogenesis. Myoepithelial cell lines (HMS-1–6), derived from diverse benign myoepithelial tumors, all constitutively express high levels of active angiogenic inhibitors, which include maspin, TIMP-1, thrombospondin-1, and soluble bFGF receptors but very low levels of angiogenic factors [Nguyen et al., 2000] (Fig. 10.3A). Recently, maspin has been shown conclusively to be an angiogenesis inhibitor [Zhang et al., 2000]. As expected, our myoepithelial cell lines inhibited endothelial cell chemotaxis (Fig. 10.3B) and proliferation. These myoepithelial cell lines sense hypoxia and respond to low O_2 tension by increased hypoxia-inducible factor-1α (HIF-1α) but with only a minimal increase in VEGF and nitric oxide synthetase (iNOS) steady-state mRNA levels. Their corresponding xenografts (HMS-X-6X) grow very slowly (Fig. 10.3C) compared with their non-myoepithelial carcinomatous counterparts and accumulate an abundant extracellular matrix devoid of angiogenesis but containing bound angiogenic inhibitors. These myoepithelial xenografts exhibit only minimal hypoxia but extensive necrosis compared with their non-myoepithelial xenograft counterparts. These former xenografts inhibit local and systemic tumor-induced angiogenesis and metastasis, presumably from their matrix-bound and released circulating angiogenic inhibitors. These observations collectively support the hypothesis that the human myoepithelial cell (even when transformed) is a natural suppressor of angiogenesis.

Myoepithelial cells in situ separate epithelial cells from stromal angiogenesis, and this seemingly banal observation serves to illustrate the fact that stromal angiogenesis never penetrates this myoepithelial barrier and raises the hypothesis that myoepithelial cells are natural suppressors of angiogenesis. This observation was reinforced by a microscopic, immunohistochemical, and DNA analysis of our myoepithelial xenografts. Our diverse myoepithelial xenografts secrete and accumulate an abundant extracellular matrix, which is devoid of blood vessels in routine hematoxylin and eosin staining and vWf immunocytochemical staining in contrast to non-myoepithelial xenografts, which show bursts of blood vessels. Microscopic quantitation of vessel density in 10 high power fields reveals absent to low vessel density in the myoepithelial xenografts compared to the non-myoepithelial xenografts ($p < 0.01$) (Fig. 3D). As mentioned previously, murine DNA Cot-1 analysis further reveals the absence of a murine component in the myoepithelial xenografts. Because, in the xenografts, angiogenesis would be murine in origin, the absence of a mur-

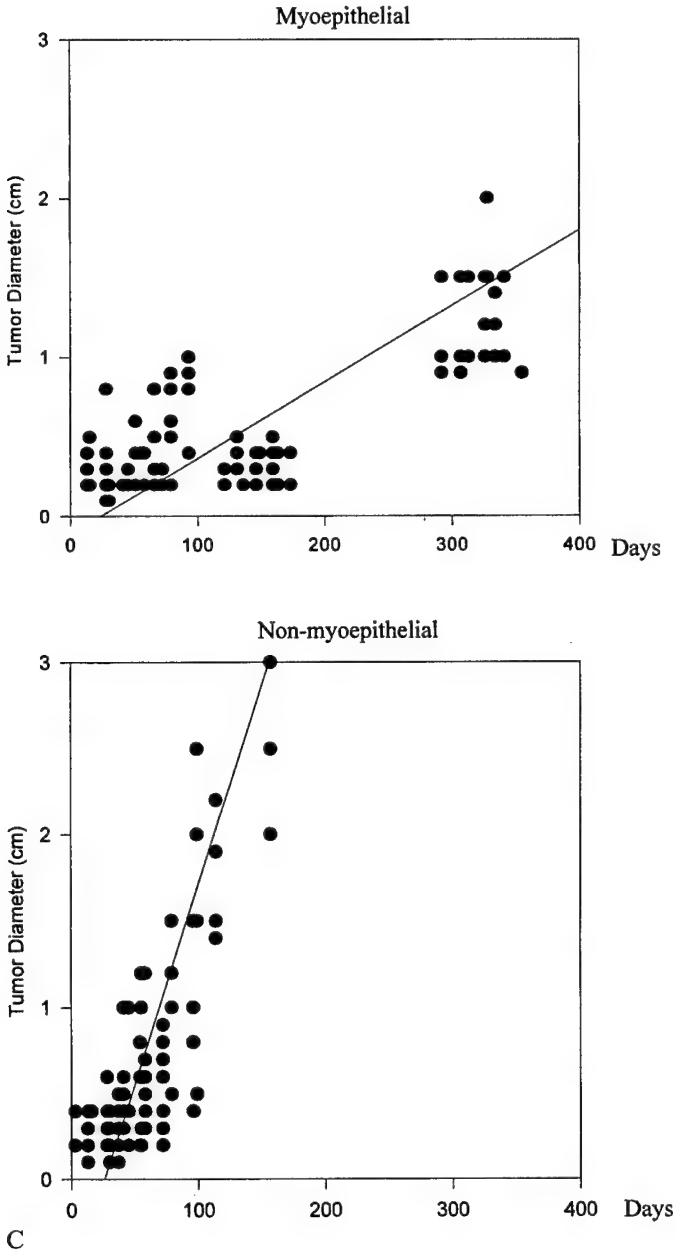


A



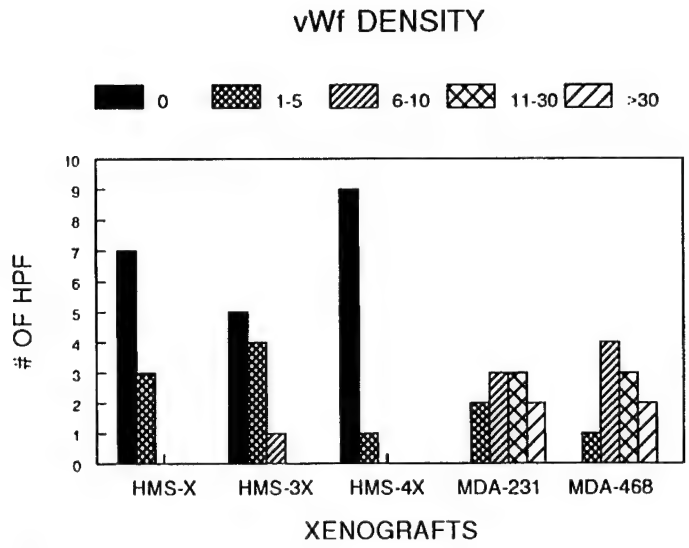
B

Figure 10.3. A) Relative constitutive gene expression profiles of diverse angiogenic inhibitors and angiogenic factors in HMS-1 compared with numerous other non-myoepithelial cell lines. All measurements were made by Western blot on either CM or HMS-X matrix extracts* and



C

Figure 10.3. *(Continued)*
depicted as relative levels of expression. HMS-1 (HMS-X*) uniquely expressed a balance of angiogenic inhibitors over angiogenic factors. B) Inhibition of HUVEC chemotaxis to bFGF by HMS-1 cells and HMS-1 CM is depicted as cell counts collected on the undersurface of a dividing filter. HMS-1 cells themselves were non-migratory. A control non-myoepithelial human melanoma cell line, M15, did not inhibit UVE chemotaxis. C) Growth rates of myo-



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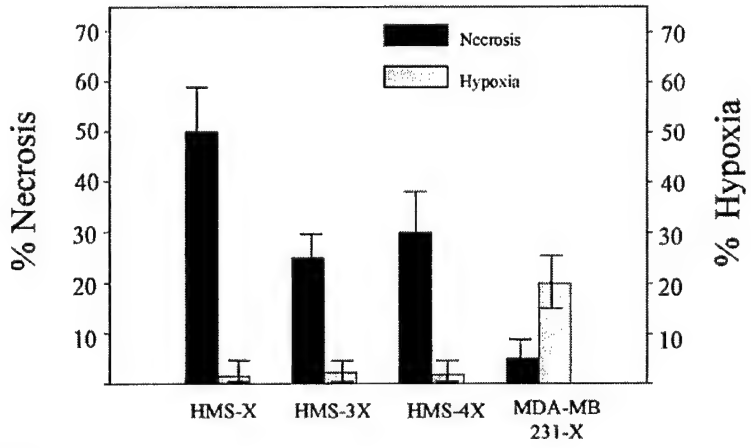
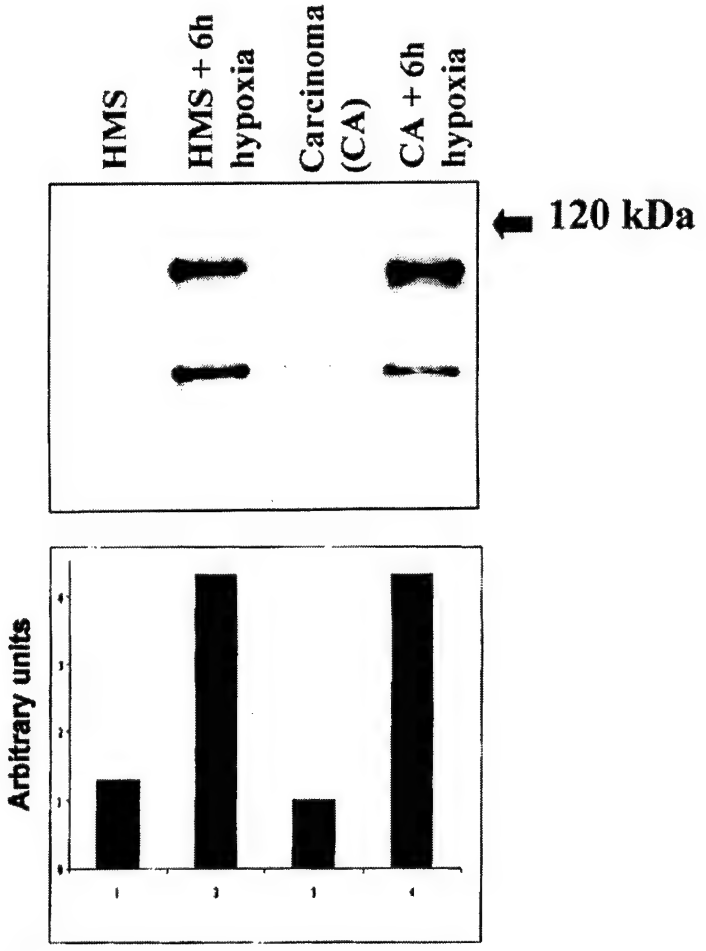


E

Figure 10.3. *(Continued)*
epithelial xenografts, for example, HMS-X, compared with growth rates of non-myoepithelial xenografts, for example, MDA-MB-231, revealed comparatively slow myoepithelial growth. This finding suggests a link to endogenously low levels of angiogenesis. D) Density of vWf-positive vessels in 10 H.P.F.'s of myoepithelial versus non-myoepithelial xenografts reveals absent-to-significantly fewer blood vessels in the former xenografts. E) Percentages of hypoxia and percentages necrosis in the myoepithelial versus non-myoepithelial xenografts are contrasted. In the myoepithelial xenografts, necrosis is prominent; whereas, hypoxia is inconspicuous where the reverse is true in the non-myoepithelial xenografts. Under low-O_2 tension, myoepithelial cells; for example, HMS-1 (HMS), like non-myoepithelial carcinoma cells; for example, MDA-MB-231 (CA), show an increase in HIF-1α (F) but, unlike carcinoma cells (CA), show less of an increase in VEGF (G) and iNOS (H) steady-state mRNA levels. Other myoepithelial and carcinoma lines tested exhibited a similar pattern of findings.

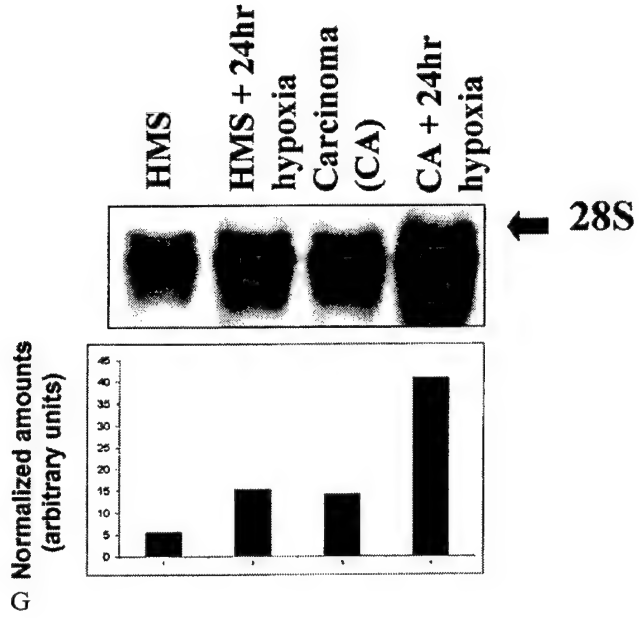


F

Figure 10.3. *(Continued)*

ine DNA component is another indication that angiogenesis is minimal. As also mentioned, the myoepithelial xenografts grew slowly compared with the non-myoepithelial xenografts, a feature that was not found in comparison between the myoepithelial versus the non-myoepithelial cell lines in vitro.

To explain these in vivo observations, we analyzed the gene expression profiles of our myoepithelial cell lines versus non-myoepithelial cell lines with respect to known angiogenic inhibitors and angiogenic factors. HMS-1, as a prototype myoepithelial cell line, constitutively expressed none of the known angiogenic factors, including bFGF, aFGF, angiogenin, TGF-α, TGF-β, TNF-α, VEGF, PD-ECGF, pla-



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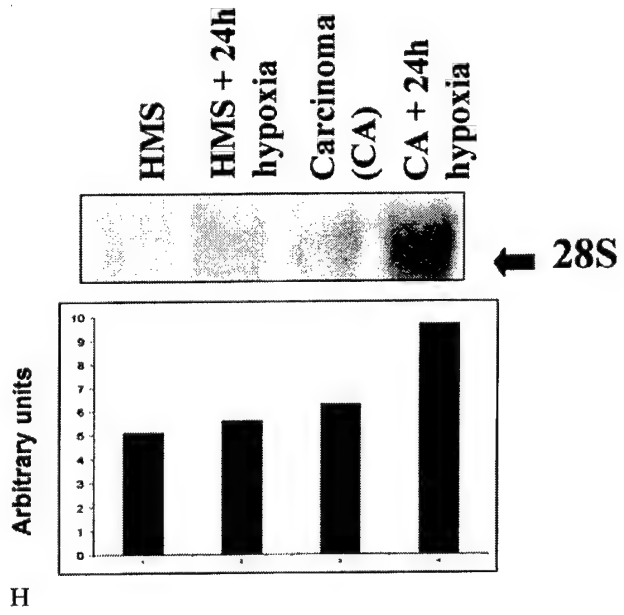


H

Figure 10.3. *(Continued)*

cenal growth factor (PlGF), IFα, HGF, and HB-EGF, but rather expressed maspin, thrombospondin-1, TIMP-1, and soluble bFGF receptors at high levels; this was in contrast to a high angiogenic factor (which included bFGF, VEGF, TGF-α, TGF-β, HB-EGF, and PD-ECGF) to angiogenic inhibitor gene expression profile, which was observed in non-myoepithelial cell lines.

Other myoepithelial cell lines (HMS-2–6) exhibited an angiogenic inhibitor/angiogenic factor profile similar to that of HMS-1. Interestingly, in 2 M urea extracts of the myoepithelial xenografts, but not in any of the non-myoepithelial xenografts, strong thrombospondin-1, TIMP-1, as well as plasminogen and prolactin fragments could be detected by Western blot. HMS-1 and HMS-1 CM (concentrated 10- to 100-fold) exerted a marked inhibition of endothelial migration (Fig. 10.3B) and proliferation, both of which were abolished by pretreatment of the myoepithelial cells with cycloheximide or dexamethasone. HMS-1 cells themselves did not migrate in response to either K-SFM, FCS, or bFGF.

When mixed with HUVEC, HMS-1 cells reduced endothelial migration to 12% $\pm$ 6% of control ($P < 0.01$) (Fig. 10.3B). HMS-1 concentrated CM reduced migration to 8% $\pm$ 7% of control ($P < 0.01$). All of the non-myoepithelial malignant human cell lines studied stimulated both endothelial migration and proliferation. Concentrated CM from HMS-1, when fractionated on a heparin-Sepharose column, inhibited endothelial proliferation to 47% $\pm$ 10% of control ($P < 0.01$). This inhibitory activity was present only in the 1.5–2.0 M gradient fraction. Pretreatment of HMS-1 cells with PMA resulted in a two- to fivefold increase in endothelial antiproliferative inhibitory activity in both unfractionated CM, as well as in the heparin-Sepharose fraction. Western blot of the heparin-Sepharose column fractions revealed that the 1.5–2.0 M NaCl fraction contained thrombospondin-1. Immunoprecipitation of this fraction with anti-thrombospondin was effective at removing all thrombospondin-1, but it decreased endothelial antiproliferative activity by only 50% and raised the possibility that other angiogenic inhibitors, including maspin, were present in this fraction. The other myoepithelial cell lines (HMS-2–6) exhibited similar anti-angiogenic inhibitory activity in their fractionated and unfractionated CM. Therefore, it is likely that both maspin and thrombospondin-1 are anti-angiogenesis effector molecules of myoepithelial cells.

To further explain our in vivo observations of minimal angiogenesis in our myoepithelial xenografts, we performed in vitro and in vivo hypoxia studies. Non-myoepithelial xenografts; for example, MDA-MB-231, exhibited florid hypoxia but only minimal necrosis when they reached a size of 2.0 cm (Fig. 10.3E). In contrast, the myoepithelial xenografts exhibited only minimal hypoxia but prominent ne-

crosis ($P < 0.001$) at the same size of 2.0 cm (Fig. 10.3E). Quantitation of the areas of hypoxia (pimonidazole immunoreactivity) and areas of necrosis in the myoepithelial versus non-myoepithelial xenografts suggested that, in the myoepithelial tumors where angiogenesis is minimal, hypoxic areas progress to necrosis rapidly. In the non-myoepithelial tumors, however, hypoxic areas accumulate but do not progress to necrosis, presumably as a result of the angiogenesis which the hypoxia elicits (Fig. 10.3E).

Comparative analysis of myoepithelial versus non-myoepithelial cell lines to low O_2 tension revealed that, although both cell lines sense hypoxia in that they responded by increasing HIF-1α (Fig. 10.3F), the myoepithelial lines up-regulated their steady-state mRNA levels of the downstream genes, VEGF (Fig. 10.3G) and iNOS (Fig. 10.3H) to a lesser extent than the carcinoma lines, suggesting the possibility of decreased transactivation of hypoxia response element (HRE). Specifically, we observed an approximate 1.7-fold increase in VEGF (1.1-fold increase in iNOS) in myoepithelial cells in response to hypoxia compared to an approximate 2.5-fold increase in VEGF (1.5-fold increase in iNOS) in carcinoma cell lines in response to hypoxia. Although these fold differences by themselves were not impressive, the absolute levels of VEGF (and iNOS) expressed in carcinoma cells in response to hypoxia were 2.5-fold greater for VEGF (and 1.7-fold greater for iNOS) than the levels of VEGF (and iNOS) expressed in myoepithelial cells in response to hypoxia. Therefore, it can be concluded that myoepithelial cells did not express VEGF or iNOS in response to hypoxia to nearly the same extent as carcinoma cells.

To study both local and systemic effects of myoepithelial cells on metastasis, we injected spontaneously metastasizing tumor cells into our myoepithelial xenografts. The highly metastatic *neo*C8161 cells injected into the myoepithelial xenografts could be recovered in significant numbers, although the numbers of clones recovered were less than those recovered from the non-myoepithelial xenografts. Histological analysis of the extirpated xenografts revealed *neo*C8161 cells actively invading through all of the non-myoepithelial xenografts in contrast to the appearance in the myoepithelial xenografts, where the *neo*C8161 cells were confined to the immediate areas around the injection site. Pulmonary metastases of *neo*C8161 were completely absent in the myoepithelial xenograft-injected group, whereas they were quite numerous in the non-myoepithelial group ($P < 0.001$). Analysis of extirpated myoepithelial xenografts containing injected *neo*C8161 cells showed no evidence of murine angiogenesis by either vWf immunocytochemical studies or murine DNA Cot-1 analysis, whereas a similar analysis of extirpated *neo*C8161 injected-non-myoepithelial xenografts showed an increase in murine angiogenesis by

both methods. This suggested that either the matrices of our myoepithelial xenografts or gene product(s) of the myoepithelial cells or both were inhibiting *neo*C8161-induced angiogenesis in vivo. We, in fact, found evidence of maspin, thrombospondin-1, TIMP-1, soluble bFGF receptors, prolactin, and plasminogen fragments within 2 M urea extracts of our myoepithelial xenografts.

In tail-vein injection studies of *neo*C8161 in mice harboring the myoepithelial xenografts, *neo*C8161 formed smaller pulmonary colonies than in mice harboring non-myoepithelial xenografts or in control mice (no xenografts) ($P < 0.01$). In a vWf factor immunocytochemical analysis of these smaller colonies in the mice harboring the myoepithelial xenografts, angiogenesis was minimal. These latter studies suggest the presence of circulating angiogenesis inhibitors released by the myoepithelial xenografts. Just recently we have demonstrated circulating maspin in mice harboring myoepithelial xenografts.

5. PHYSIOLOGICAL AND PHARMACOLOGICAL MANIPULATIONS

Since PMA and dexamethasone were effective at pharmacologically altering maspin levels and the myoepithelial phenotype, we wondered whether physiological agents could do so as well. Because previous basic and clinical studies had examined the role of estrogen agonists and antagonists on human breast cancer cells and because issues of hormone replacement therapy (HRT) and tamoxifen chemoprevention are such timely issues in breast cancer, we wondered whether hormonal manipulations might affect myoepithelial cells in vitro as far as their paracrine suppressive activities on breast cancer were concerned. We recently demonstrated [Shao et al., 2000] that treatment of myoepithelial cells with tamoxifen, but not 17-β estradiol, increases both maspin secretion and invasion-blocking ability. 17-β Estradiol, however, competes with these suppressive effects of tamoxifen, which suggests that the mechanism of tamoxifen action is estrogen-receptor mediated. Myoepithelial cells lack ER-α but express ER-β. Tamoxifen, but not 17-β estradiol, increases AP-1 CAT but not ERE-CAT activity. Again, 17-β estradiol competes with the transcription-activating effects of tamoxifen. These experiments collectively suggest that the actions of tamoxifen on the increased secretion of maspin by myoepithelial cells may be mediated through ER-β and the *trans*-activation of an ER-dependent AP-1 response element.

As mentioned previously, immunoprecipitation of maspin from HMS-1 CM reversed the anti-invasive effects of myoepithelial CM on breast carcinoma cell invasion in vitro. Tamoxifen treatment of HMS-1 resulted in a two- to threefold increase in maspin secretion with

increasing doses of tamoxifen and increasing times of exposure. 17-β Estradiol, in contrast, exerted no effects on maspin secretion and completely abolished the maspin stimulatory effects of tamoxifen in competition experiments. Tamoxifen's increase in maspin secretion was not due to an increase in steady-state maspin mRNA levels, which were essentially unchanged by this treatment. Myoepithelial cell lines lacked ER-α expression but uniformly expressed ER-β. Because estrogen agonists/antagonists bound to estrogen receptors (either ER-α or ER-β) activate downstream genes containing either a classical ERE or an ER-dependent AP-1 response element, myoepithelial cell lines were transfected with CAT-reporter constructs fused to heterologous promoters containing the human estrogen response element (ERE-tk-CAT) or AP-1-tk-CAT. Tamoxifen (10^{-7} M) increased AP-1-CAT activity threefold. This effect was not observed with 17-β estradiol. Furthermore 17-β estradiol (10^{-5} M) competed with and blocked the effects of tamoxifen (10^{-7} M). 17-β estradiol (10^{-7} M) did increase ERE-CAT activity but tamoxifen (10^{-7} M) did not.

6. MYOEPITHELIAL GENE PRODUCTS AS SURROGATE END-POINT MARKERS

Because myoepithelial cells are ubiquitous components of the ductal-lobular units of the breast and other organs, which exhibit branching morphogenesis, we hypothesized that gene products of myoepithelial cells might be detectable in fluid secreted by these ductal-lobular units. As there has been a lot of recent interest in breast ductal fluid and breast nipple aspirates, especially, we measured one myoepithelial gene product, maspin, by Western blot, and found it to be present in both nipple aspirates and ductal fluid but not blood or urine [unpublished observations). These observations indicate that ductal fluid is not a mere transudate of blood or serum and that it is not a product only of epithelial cells (although epithelial protein products, such as casein, lactalbumin, and carcinoembryonic antigen (CEA), are certainly present). Ductal fluid also reflects a significant contribution from myoepithelial cells. From this observation, we are currently studying groups of patients to see whether their maspin levels serve to stratify them. We are currently analyzing ductal fluid collected following cannulation and washing of selected ducts in patients with microcalcifications on screening mammography who are about to undergo either excisional or core biopsy. Paired comparisons of maspin levels in ductal fluid obtained from ducts harboring microcalcifications or DCIS and normal ducts from the same patients are also being made. Maspin levels can be correlated with the histopathology surrounding the microcalcifications. It is anticipated that some of

these patients will exhibit normal ductal histopathology surrounding their microcalcifications, some will harbor proliferations, such as hyperplasia, adenosis, ADH, and DCIS, and still others invasive carcinoma. The screening value of maspin levels in all of these patients can be determined. Measurements of myoepithelial maspin in ductal fluid will be compared with levels of a breast epithelial cell marker, such as CEA. Increased CEA has been observed in nipple secretions and in ductal fluid in patients with ductal hyperplasia. Hence, the maspin/CEA ratio might be predictive of risk with increased maspin/CEA correlating with normalcy and decreased maspin/CEA correlating with high risk, microcalcifications, and/or precancerous histopathology. In this sense, maspin can be used as a surrogate end-point marker to predict either the risk of DCIS or the likelihood that DCIS will progress to invasive breast cancer.

Another interesting observation with respect to the use of myoepithelial maspin as a marker—this time, a tumor marker—is the observation that maspin can be detected in normal saliva but that it is markedly elevated in saliva secreted from a salivary gland neoplasm and that it is also elevated in murine serum in mice harboring human myoepithelial xenografts [unpublished observations]. Most salivary gland neoplasms are thought to be myoepithelial in origin. These include mixed tumors, basal cell adenomas, basal cell adenocarcinomas, and adenoid cystic carcinomas. It was human tumors of these types that originally gave rise to our myoepithelial cell lines/xenografts that led to a dissection of the myoepithelial phenotype and to our observations concerning myoepithelial maspin. If screening saliva for maspin shows promise for detecting small incipient salivary gland neoplasms, then myoepithelial maspin will show its utility as a tumor marker. So, in summary, our findings indicate that the gene products of myoepithelial cells; for example, maspin reflect the structural and functional integrity of the ductal-lobular units of different organs, and alterations in the levels of these myoepithelial gene products in fluid from these units may reflect disease states.

7. METHODS OF CULTURING TRANSFORMED MYOEPITHELIAL CELLS

As has been shown, transformed myoepithelial cells can be derived from benign myoepithelial tumors. The most common site of myoepithelial neoplasia is the salivary glands. Myoepithelial tumors include the pleomorphic adenoma, the basal cell adenoma, the basal cell adenocarcinoma, and the adenoid cystic carcinoma. Although the cells of these tumor are transformed, karyotype analysis is very close to normalcy. Analysis of the gene products of these cells reveals minimal

differences from normal myoepithelial cells; therefore, we believe that these transformed myoepithelial cells can serve as normal myoepithelial cell surrogates. Our laboratory has been successful in establishing six different human myoepithelial cell lines and xenografts using the following methods.

8. PREPARATION OF MEDIA AND REAGENTS

8.1. Supplemented K-SFM

K-SFM keratinocyte serum-free medium supplemented with recombinant epidermal growth factor (EGF, 5 ng/ml) and bovine pituitary extract (BPE, 50 μg/ml)

8.2. Attachment medium

K-SFM with 0.5% FBS.

8.3. CMF-HBSS

Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution.

8.4. Disaggregation medium

F12:DMEM (1:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium) with 10mM HEPES buffer, 2% bovine serum albumin, fraction V, 5 μg/ml Insulin, 300 U/ml Collagenase, and 100 U/ml Hyaluronidase.

8.5. Trypsin-EDTA

Trypsin, 0.05%, 0.7 mM EDTA (disodium ethylene diamine tetraacetate) in CMF-HBSS.

8.6. F12/DMEM/H

F12/DMEM with 15 mM Hepes.

8.7. Dispase medium

F12/DMEM/H with a reduced Ca^{2+} concentration (0.06 mM), and containing 5 U/ml dispase.

8.8. Serum-free F12/DMEM/H

F12:DMEM:H medium supplemented with 1 mg/ml BSA, 1 g/ml insulin, 0.5 g/ml hydrocortisone, 10 ng/ml cholera toxin, 10 ng/ml epidermal growth factor.

8.9. High-salt buffer

3.4 M NaCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM N-ethylmaleimide (NEM) pH 7.4.

8.10. Urea/guanidinium-HCl extraction buffer

6 M urea, 2 M guanidinium-HCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM NEM, pH 7.4, with added 2.0 mM dithiothreitol (DTT).

8.11. Tris buffered saline (TBS)

0.15 M NaCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM NEM, pH 7.4.

Protocol 10.1. Culture of Transformed Human Myoepithelial Cells

Reagents and Materials

Sterile

- Supplemented K-SFM: (see Section 8.1)
- Trypsin-EDTA: (see Section 8.5) Attachment medium: (see Section 8.2)
- CMF-HBSS: (see Section 8.3)
- Dimethylsulfoxide (DMSO)
- Culture dishes, 5 cm
- Culture flasks, 25 cm^2 Scalpels, #11 blade
- Cryovials

Protocol

(a) After human subject consent and approval of the institutional review board, obtain tissues following surgery.

(b) Mince portions of the surgical specimen to ~1 mm^3 under aseptic conditions.

(c) Transfer to dishes containing supplemented K-SFM. Have sufficient medium in the dishes so that the explants are in contact with medium but are able to adhere to the bottom of the dish without being dislodged.

(d) Culture the cells at 37°C in a humidified atmosphere at 5% CO_2 in air.

(e) Change the medium every third day. After 1–2 weeks, cells migrate out from the explants. The medium is suppressive to fibroblast growth but stimulatory to myoepithelial growth.

(f) For subculture of cell monolayers, wash inCMF-HBSS, and detach with trypsin-EDTA.

(g) Resuspend trypsinized cells in attachment medium and allow to attach overnight.

(h) Replace medium with serum-free supplemented K-SFM and maintain thereafter in this serum-free growth medium. Cells are generally seeded in tissue culture flasks at 1/6–1/12 of the confluent density or 0.5–1 × 10^4 cells/cm^2.

(i) Prepare frozen stocks in serum-free medium containing 10% DMSO.

If there is difficulty in establishing the cell line in this manner, you can transplant the initial tumoral explants subcutaneously in the flanks of female nude (nu/nu mutants on a BALB/c background) or SCID mice with a number 10 trochar. We have had a much higher success rate (virtually 100%) with xenograft establishment compared with cell line establishment (10% success rate). The xenografts are very slow growing, however, reaching approximately 1 cm in diameter after 6 months to 1 year (Fig. 10.3C). When the xenografts reach this size, they can be removed by sterile technique, minced, and transplanted to new mice. Portions can also be slow-frozen in serum-free medium containing 10% DMSO and stored in liquid nitrogen. At any point, attempts may again be made to establish a cell line by placing the xenograft explants in cell culture in a manner identical to that for the initial surgical specimen. Using this approach, the success rate for establishing a cell line can be doubled. These strategies are summarized in the Schematic of Figure 10.4.

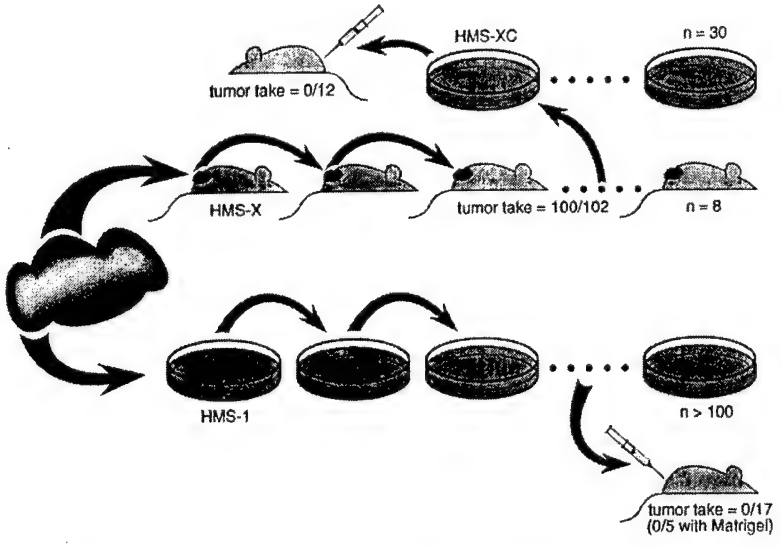


Figure 10.4. Schematic depicts successful method for obtaining myoepithelial cell lines (albeit transformed) from benign myoepithelial tumors of the salivary gland and breast.

9. METHODS OF CULTURING NORMAL MYOEPITHELIAL CELLS

There is no doubt that the use of transformed myoepithelial cells, however convenient, has limitations. For one, these immortalized myoepithelial cells are highly selected and represent homogeneous cell populations, which may not accurately reflect the heterogeneous composition of myoepithelial cells within the normal human mammary gland. Also, transformed myoepithelial cell lines are likely to have acquired genetic changes in long-term culture that confound analysis of the properties of the original myoepithelial cells of the breast or other primary organ. For these reasons, methods to obtain and culture primary normal myoepithelial cells would also be desirable.

Normal breast epithelium consists of different cell types, including luminal epithelial cells that line the ducts and alveoli and basally located myoepithelial cells. These two cell types can be distinguished on the basis of expression of distinct cell markers. The most commonly used phenotypic markers to identify luminal epithelial cells are the MUC-1 apical plasma membrane glycoprotein and cytokeratins 8, 18, and 19. For myoepithelial cells, markers include the common acute lymphocytic leukemia antigen (CALLA/CD10), smooth-muscle actin, cytokeratins 5, 14, and 17, S100, calponin, and maspin. Most studies examining mammary cells in primary culture have utilized the heterogeneous mixed population of epithelial cells (the total epithelial cell population) in the tissue sample. Studies indicate that this heterogeneous mixed population of epithelial cells really consists of luminal epithelial cells and basal myoepithelial cells (see also Chapter 9). Similarly, commercially available HMEC (Clonetics) really consist of both epithelial as well as myoepithelial cells.

Protocol 10.2. Culture of Normal Human Myoepithelial Cells

Reagents and Materials

Sterile

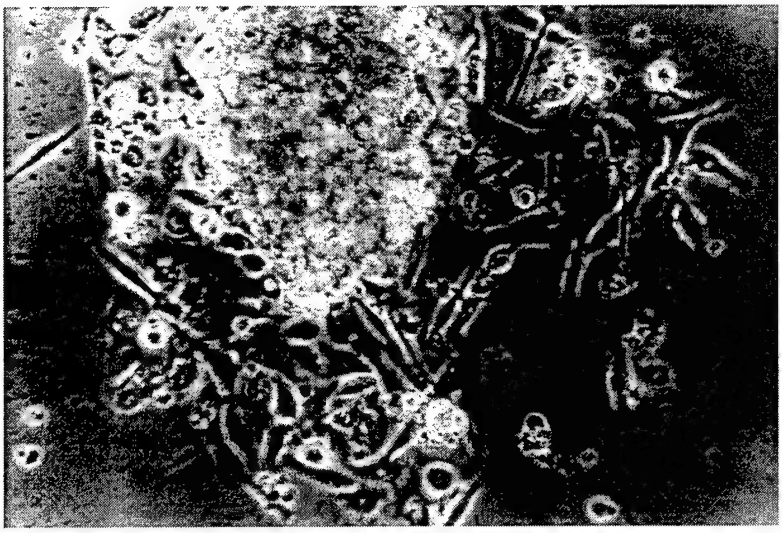
- Disaggregation medium: (see Section 8.4)
- F12/DMEM/H (see Section 8.6) with 5% FCS
- Serum-free F12/DMEM/H (see Section 8.8) or supplemented K-SFM (se Section 8.1)
- Dispase medium: (see Section 8.7)
- Trypsin/EDTA (see Section 8.5)
- DMSO
- Nylon mesh, 20 μm
- Collagen-coated tissue culture dishes or plastic flasks
- Cryovials

Protocol

(a) After human subject consent and approval of an institutional review board, obtain mammary gland tissues (mainly from reduction mammoplasties) following surgery.
(b) After tissue is taken for adequate diagnosis, dissociate the remainder of the tissue and culture the epithelial cells [Emerman et al., 1990; Emerman et al., 1994] as follows.
(c) Trim fat from tissue samples and mince the tissue.
(d) Dissociate by shaking at 37°C for 18 h in disaggregation medium.
(e) Collect the epithelial cell pellet by centrifuging the cell suspension at 80 × g for 4 min.
(f) If a single cell suspension is required; for example, for FACS-sorting, resuspend the cells in trypsin/EDTA for 4 min.
(g) Add F12/DMEM/H containing 5% FCS to inhibit the enzymatic activity of trypsin.
(h) Filter the resultant cell suspension through 20 μm nylon mesh
(i) Centrifuge the filtrate at 100 × g for 5 min, and resuspend the pellet in dispase medium to prevent reaggregation of the cells.
(j) Count the number of viable cells, determined by Trypan blue exclusion, on a hemocytometer.
(k) Seed cells onto collagen-coated tissue culture dishes or plastic flasks.
(l) Culture overnight in F12/DMEM/H containing 5% FCS to allow attachment of cells.
(m) Switch the medium to serum-free F12/DMEM/H or supplemented K-SFM. The purpose of this initial phase of culture is to allow the "total epithelial cells" to adhere and to allow for the selective removal of non-attaching contaminating cells and debris.
(n) At 80% confluence, harvest cells via trypsinization, wash, and freeze in liquid nitrogen as viable cell suspensions.

The average epithelial cell numbers obtained from dissociated normal, dysplastic, and malignant human breast tissue are: normal, 0.5×10^6 cells/g tissue; fibroadenoma, 1.5×10^6 cells/g tissue; fibrocystic, 0.7×10^6 cells/g tissue; carcinoma, 1.0×10^6 cells/g tissue. Cell numbers are increased by culturing. Cell numbers of 5×10^6–1×10^7 can be obtained from primary and early passage cultures, providing sufficient material for experimental manipulations and analyses.

The harvested cells can be analyzed in either two-dimensional or three-dimensional culture systems, both of which reveal two populations of cells: epithelial cells with luminal characteristics and myoepithelial cells with characteristic myoepithelial markers [Yang et al., 1986; Rudland, 1991]. Using either two-dimensional or three-



A

MIXED CELL POPULATION GROWING IN KSFM

MUC-1 + CALLA- luminal EPITHELIAL CELL

MUC-1-CALLA+ basal MYOEPITHELIAL CELL

Two dimensional: tightly arranged luminal cells

Two dimensional: teardrop shaped myepithelial cells

Three dimensional: small spherical alveolar-like colonies

Three dimensional: large branched ductal-like colonies; homogeneous cell types

B

dimensional culture systems, we can detect clonal populations in cultures initiated at low cell densities (<2500 cells/cm^2) with colony yields linearly related to the concentration of cells initially plated. Using either system, we can obtain clonal populations of either cell type at an efficiency of 1–3%. Most colonies generated are composed of 2–10 cells after 14 days, but colonies >50 cells are observed occasionally.

In two-dimensional culture systems; for example, on plastic, the colonies exhibit two major morphologies, which include balls of tightly arranged cells and loosely arranged tear-drop shaped cells having very distinct cell borders. Immunocytochemical analysis of the different colonies has shown that, in the colonies composed of tightly arranged cells, the cells express typical luminal epitopes (MUC-1, keratins 8, 18, and 19) but do not express the myoepithelial marker keratin 14 [Dairkee et al., 1988]. For the colonies composed of the loosely arranged tear-drop shaped cells, the cells express keratin 14, CALLA, smooth-muscle actin, maspin, calponin, S100, and other myoepithelial markers, but do not express the epithelial markers, MUC-1 or keratin 19. These dual populations of cells can be sorted by FACS to yield relatively pure populations of each cell type [O'Hare et al., 1991]. After sorting, the MUC-1$^+$, CALLA$^-$ subpopulation generates further colonies composed of tightly arranged cells. We believe that these are epithelial cells. After sorting, the MUC-1$^-$, CALLA$^+$ subpopulation generates colonies of dispersed teardrop-shaped cells. We think that these cells are myoepithelial cells. Sometimes, in the initial non-sorted population, cells expressing epithelial morphologies and cells expressing myoepithelial morphologies co-exist within a single colony (Fig. 10.5A), with the tightly arranged "luminal" cells in the center of the colony and the teardrop-shaped "myoepithelial" cells radiating out into the periphery of the colony analogous to the in vivo situation.

In three-dimensional culture systems; for example, Vitrogen and *Humatrix*, the mixed population of cells that gives rise to two populations on two-dimensional systems also gives rise to two populations in three-dimensional systems. The mixed population of cells is seeded at low cell density (<1000 cells/ml of gel) within the gel and is

Figure 10.5. (A) Primary cultures of total breast epithelial cells reveal a clump of cuboidal luminal epithelial cells surrounded by more spindly or tear-dropped myoepithelial cells in periphery. These latter cells were CALLA+ and MUC1–. (B) Schematic depicts successful method for obtaining primary myoepithelial cells from human mammary explants and identifying myoepithelial cells out of a mixed cell population based on two- and three-dimensional appearances in tissue culture and confirmatory immunocytochemistry.

cultured in serum-containing medium for 10–14 days. Colonies of varying morphologies emerge with a frequency of ~2%. Colony morphologies include small (<50 cells) spherical colonies composed of a simple cuboidal epithelium surrounding a central lumen, as well as larger (>100 cells) highly branched colonies composed of a solid cord of cells. Immunocytochemical analysis of the colonies generated in three-dimensional systems reveals that the small spherical colonies generally exhibit typical luminal epitopes (MUC-1$^+$, keratins 8/18$^+$, CALLA$^-$, keratin 14$^-$). The larger branched colonies, however, are mainly positive for myoepithelial markers (keratin 14$^+$, CALLA$^+$) and negative for luminal markers (MUC-1$^-$, keratin 19$^-$). Sorting by FACS of the mixed cell populations before seeding within gels (Vitrogen or *Humatrix*) demonstrates that the small spherical colonies derive from the MUC-1$^+$, CALLA$^-$ subpopulation (epithelial cells), and the large branched colonies derive from the MUC-1$^-$, CALLA$^+$ subpopulation (myoepithelial cells). Interestingly, it should be recalled that our transformed myoepithelial cell lines; e.g., HMS-1 give evidence of branching and budding when grown in gels, evidence that they retain this basic myoepithelial characteristic.

The schematic diagram (Fig. 10.5B) summarizes the method for obtaining and identifying myoepithelial cells from human mammary tissues with both two-dimensional and three-dimensional systems.

10. METHODS OF OBTAINING MYOEPITHELIAL MATRIX

Human myoepithelial matrix, termed *Humatrix*, can be obtained from our myoepithelial xenografts [Kedeshian et al., 1998]. This matrix is very rich in proteoglycans and hyaluronic acid (Fig. 10.6A). The method of extraction is as follows.

Protocol 10.3. Preparation of Human Myoepithelial Matrix

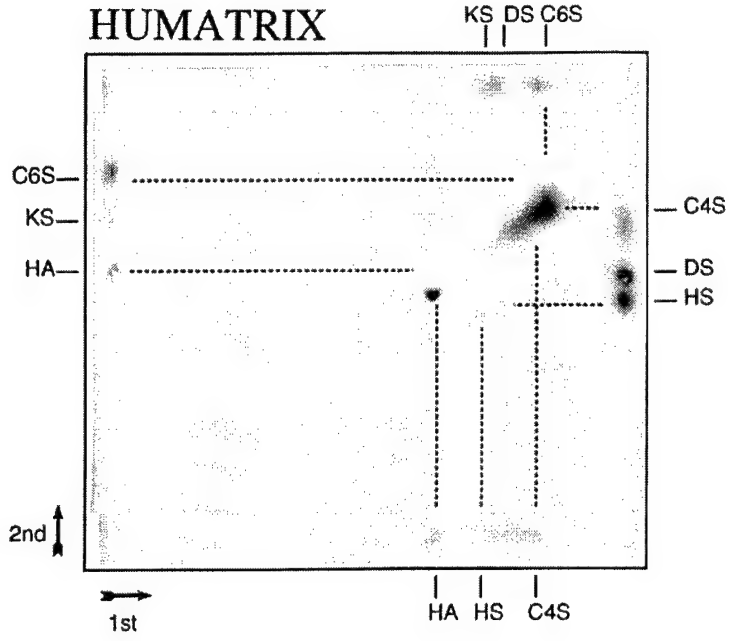
Reagents and Materials

Sterile

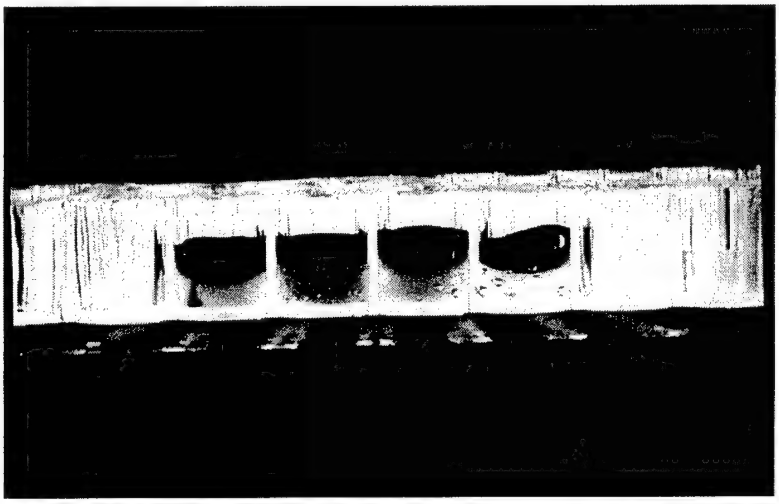
- 0.5% chloroform and cell culture medium

Non-sterile

- β-aminoproprionitrile (BAPN) fumarate fed mice High-salt buffer (see Section 8.9)
- Urea/guanidinium-HCl extraction buffer (see Section 8.10)
- Tris buffered saline (TBS) (see section 8.11)
- Dialysis membrane with a molecular weight cutoff of 5–10 kD

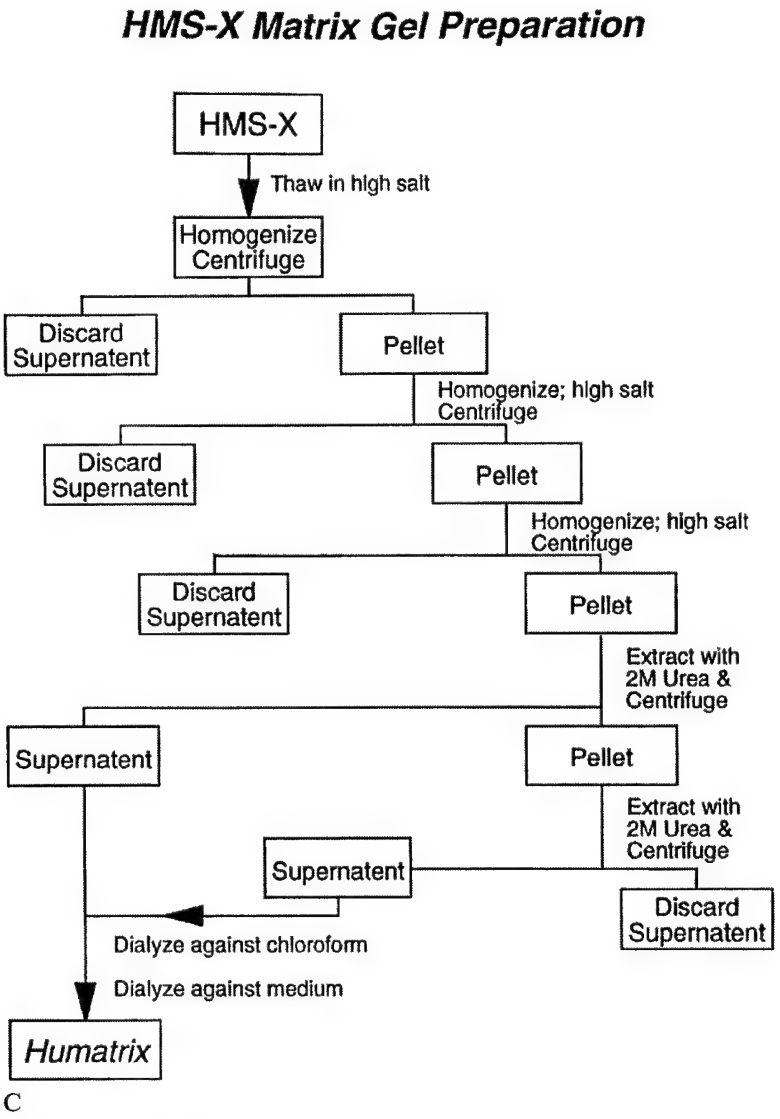


A



B

Figure 10.6. (A) Characteristics of the matrix extract from a human myoepithelial xenograft, HMS-X, is depicted in this 2-D cellulose acetate preparation. Large amounts of chondroitin-4-sulfate proteoglycan, heparan sulfate proteoglycan and hyaluronic acid are evident. (B) This matrix extract undergoes gelation at 25–37°C and excludes cells and large macromolecules. (C) Schematic depicts method of preparation of this human myoepithelial matrix gel, termed *Humatrix*.



C

Figure 10.6. *(Continued)*

Protocol

(a) Grow the tumors in -aminoproprionitrile (BAPN) fumarate fed mice, which are rendered lathyritic.
(b) Harvest HMS-X, HMS-3X, or HMS-4X tumors at 1–2 g of size.
(c) Homogenize 10 g of tumors in 2 ml/g , pH 7.4, at 4°C.
(d) Spin the homogenate for 15 min at 12,000 g at 4°C.

(e) Extract the pellet overnight at 4°C in 0.5 ml urea/guanidinium-HCl extraction buffer per g starting material with gentle stirring.
(f) Spin the extract for 30 min at 24,000 g at 4°C.
(g) Dialyze the supernatant against several changes of TBSat 4°C, followed by sequential dialyses against 0.5% chloroform in cell culture medium by using a dialysis membrane with a molecular weight cutoff of 5–10 kD.
(h) Using the urea/guanidinium-HCl method, 1 ml of *Humatrix* at a protein concentration of 1.5 mg/ml is obtained from each g of HMS-X.
(i) Concentrate the final solution to 3 mg/ml protein by ultrafiltration at 4°C.
(j) Store *Humatrix* at −20°C. At 4°C, *Humatrix* remains liquid; at 25 −37°C *Humatrix* undergoes gelation (Fig. 10.6B).

Humatrix can also be prepared by a pepsin hydrolysis method similar to that used in the preparation of Vitrogen 100. This method of human myoepithelial matrix extraction is summarized in the following schematic (Fig. 10.6C).

11. FUTURE MYOEPITHELIAL RESEARCH DIRECTIONS

The observations that myoepithelial cells secrete suppressive gene products, such as maspin, in large quantities, whereas carcinoma cells do not, suggest that myoepithelial cells exert pleiotropic suppressive effects on tumor progression. Because these gene products are proteinase inhibitors, locomotion inhibitors, and angiogenesis inhibitors, their diverse actions may largely explain the pronounced anti-invasive and anti-angiogenic effects of myoepithelial cells on carcinoma and pre-carcinoma cells. Clearly, the gene products of myoepithelial cells have more than marker value. We need to better understand what it is about the myoepithelial phenotype that allows for high constitutive expression and secretion of tumor suppressive molecules. Studies of the maspin promoter and *cis/trans* interactions within the myoepithelial cell seem to be an attractive line of further research. We also need to understand better the mechanism by which certain pharmacological agents, such as PMA, and certain physiological agents, such as tamoxifen, bolster myoepithelial secretion of suppressive molecules, such as maspin. With this understanding we may be able to design smaller, less-toxic molecules that have the same effect. We need to exploit better the intricate paracrine and local relationships that exist between myoepithelial cells and epithelial cells (precancerous and cancerous) in the breast and other organs. This point is especially im-

portant and timely as intraductal approaches through the nipple are gaining in popularity as a means of screening women who are at risk for developing breast cancer. These intraductal approaches really exploit the local myoepithelial/epithelial relationships that exist. Screening for maspin levels as a surrogate end-point marker is only the beginning. One could envision delivering intraductal gene therapy designed to exploit the inherent differences between myoepithelial and epithelial cells. One could target and destroy the epithelial cells, selectively sparing the myoepithelium or alternately target the myoepithelial cells with a vector, which bolsters its secretion of suppressive molecules. If the myoepithelial defense can be bolstered in this manner, perhaps this natural barrier, which normally inhibits invasion for years, can be made into an impervious barrier, which inhibits invasion forever. At least that is one vision of scientists who are interested in myoepithelial cells.

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SOURCES OF MATERIALS

Item	Supplier
Bovine pituitary extract	Gibco
BSA, fraction V	Gibco
Collagenase	Sigma
Collagen-coated tissue culture dishes or plastic flasks	Collaborative Biomedical Products (BD Biosciences)
Cryovials	Nalge Nunc
Culture dishes and flasks	BD Biosciences
Dispase	Collaborative Biomedical Products (BD Biosciences)
EDTA	Sigma
EGF	Gibco
FCS	Gibco
Ham's F12: Dulbecco's modified Eagle's medium (F12:DMEM)	Stem Cell Technologies Inc.
HEPES	Sigma
Hyaluronidase	Sigma
Insulin	Sigma
K-SFM keratinocyte serum-free medium	Gibco
Nylon mesh	BioDesign
Recombinant EGF	Gibco
Trypsin	Gibco
Vitrogen	Nutacon

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(12) **United States Patent**
Barsky et al.

(10) **Patent No.: US 6,514,695 B1**
(45) **Date of Patent: Feb. 4, 2003**

(54) **COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THERAPY**

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(73) Assignee: **The Regents of the University of California**, Oakland, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/488,464**

(22) Filed: **Jan. 20, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/116,470, filed on Jan. 20, 1999.

(51) **Int. Cl.**[7] **C12Q 1/68**; C12N 15/63; C12N 15/85; C07H 21/04

(52) **U.S. Cl.** **435/6**; 435/320.1; 435/455; 536/23.1

(58) **Field of Search** 435/6, 320.1, 455; 514/44; 536/23.1

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(74) *Attorney, Agent, or Firm*—Gates & Cooper LLP

(57) **ABSTRACT**

The present invention provides methods for the selective transduction of a cell in a ductal system in a mammary gland by contacting, via ductal cannulation, the cell with a vector that selectively targets the cell. In this context, the invention provides prophylactic and therapeutic methods of treating the ductal epithelium of the breast, for disease, in particular cancer. The present invention further provides diagnostic methods of determining the presence of disease in the ductal epithelium of the breast, in particular cancer.

12 Claims, 8 Drawing Sheets

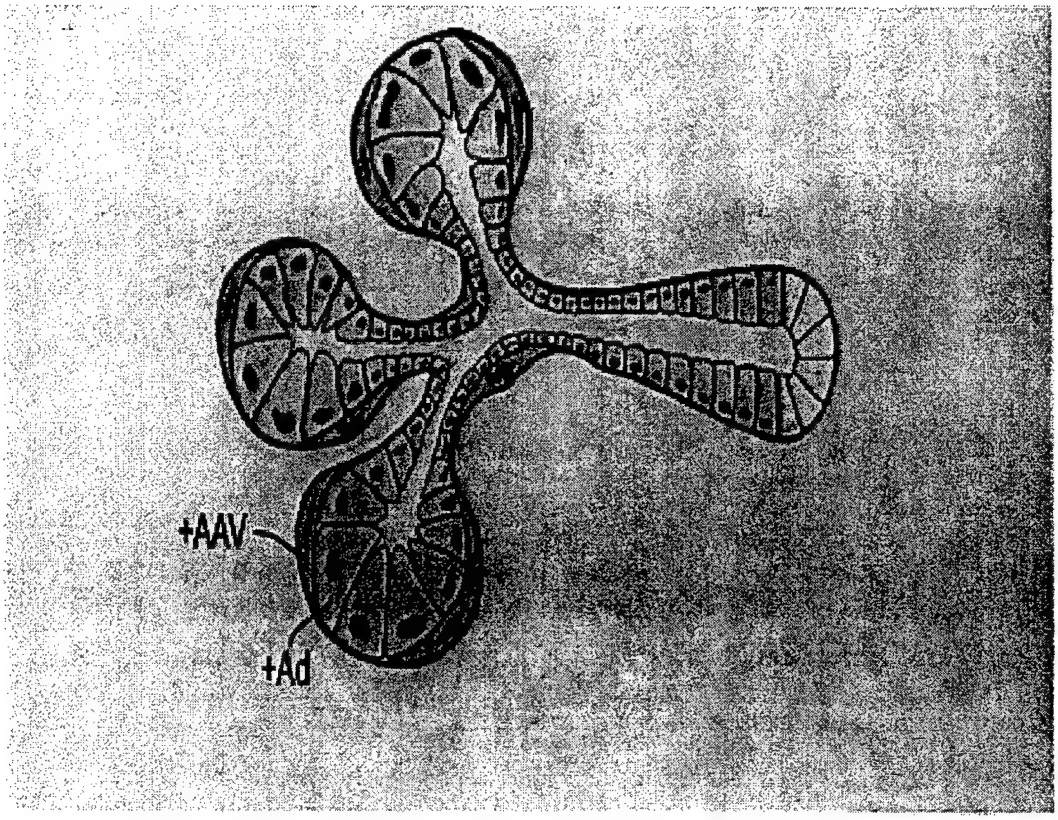


FIG.1

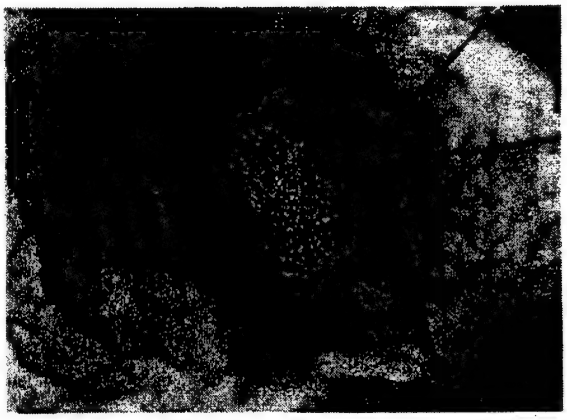


FIG. 2A

FIG. 2B

FIG. 3A

FIG. 3B

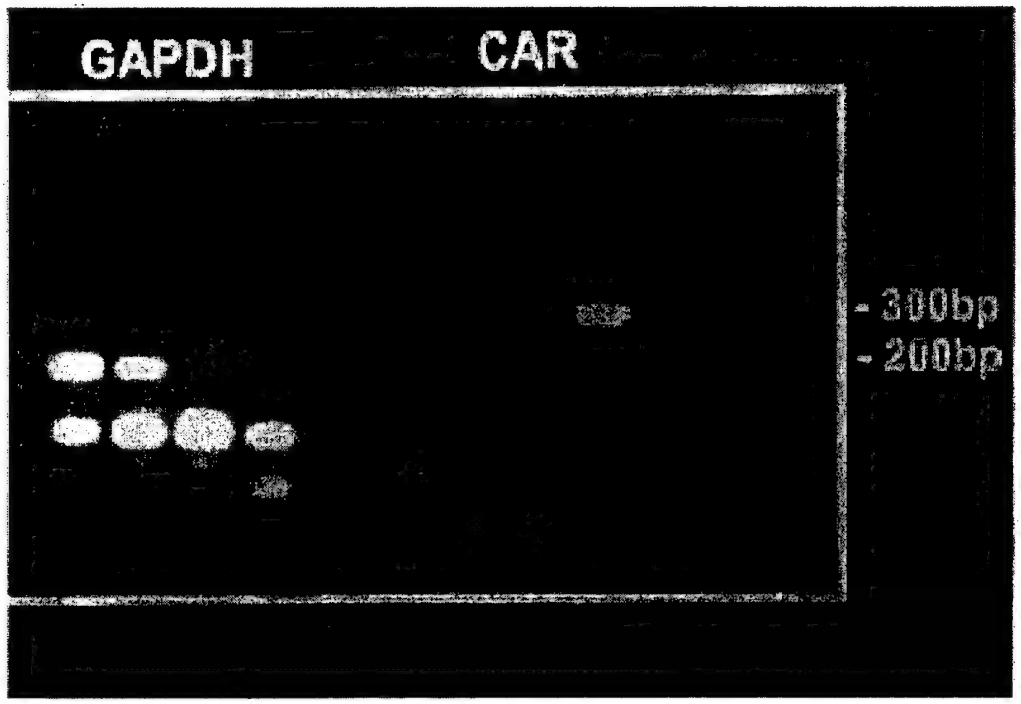


FIG. 3C

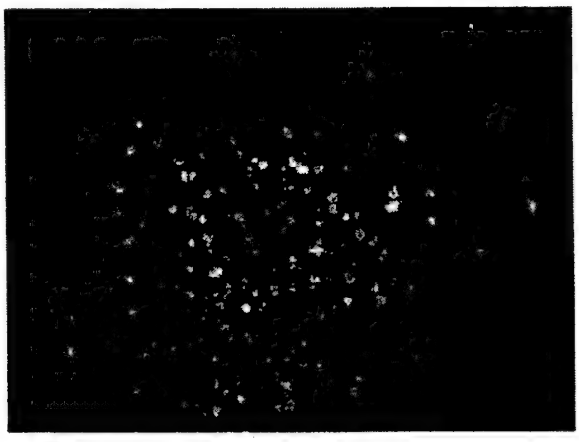


FIG. 4A

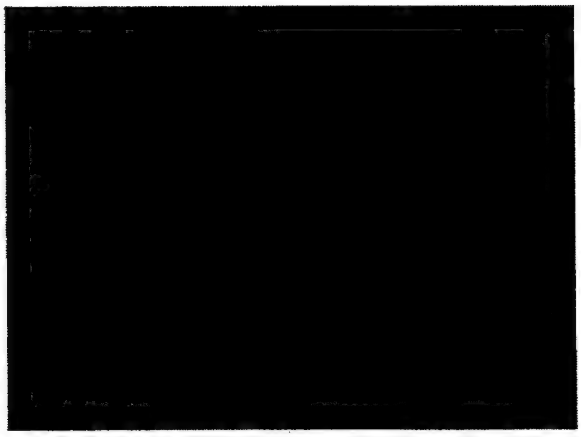


FIG. 4B

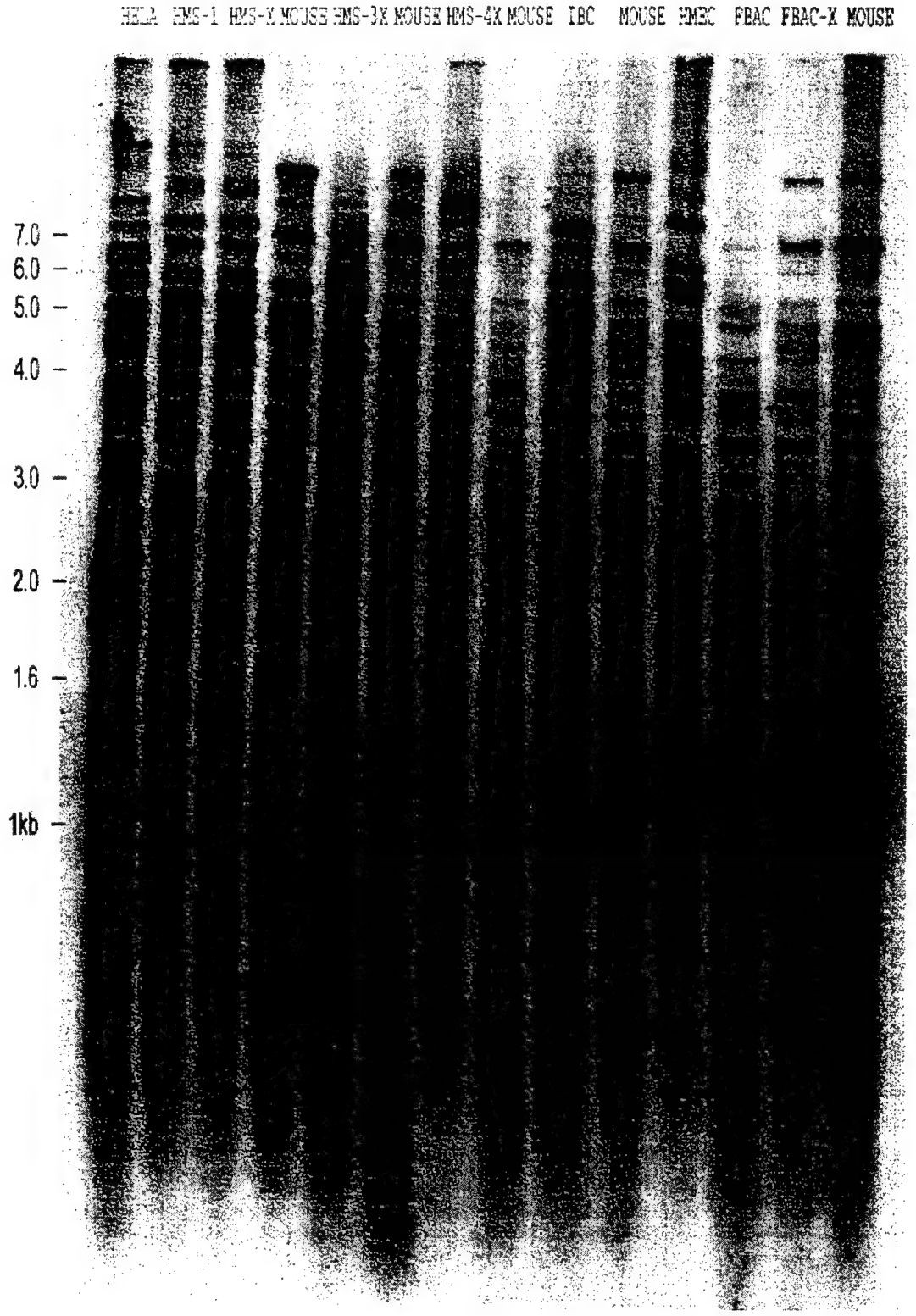
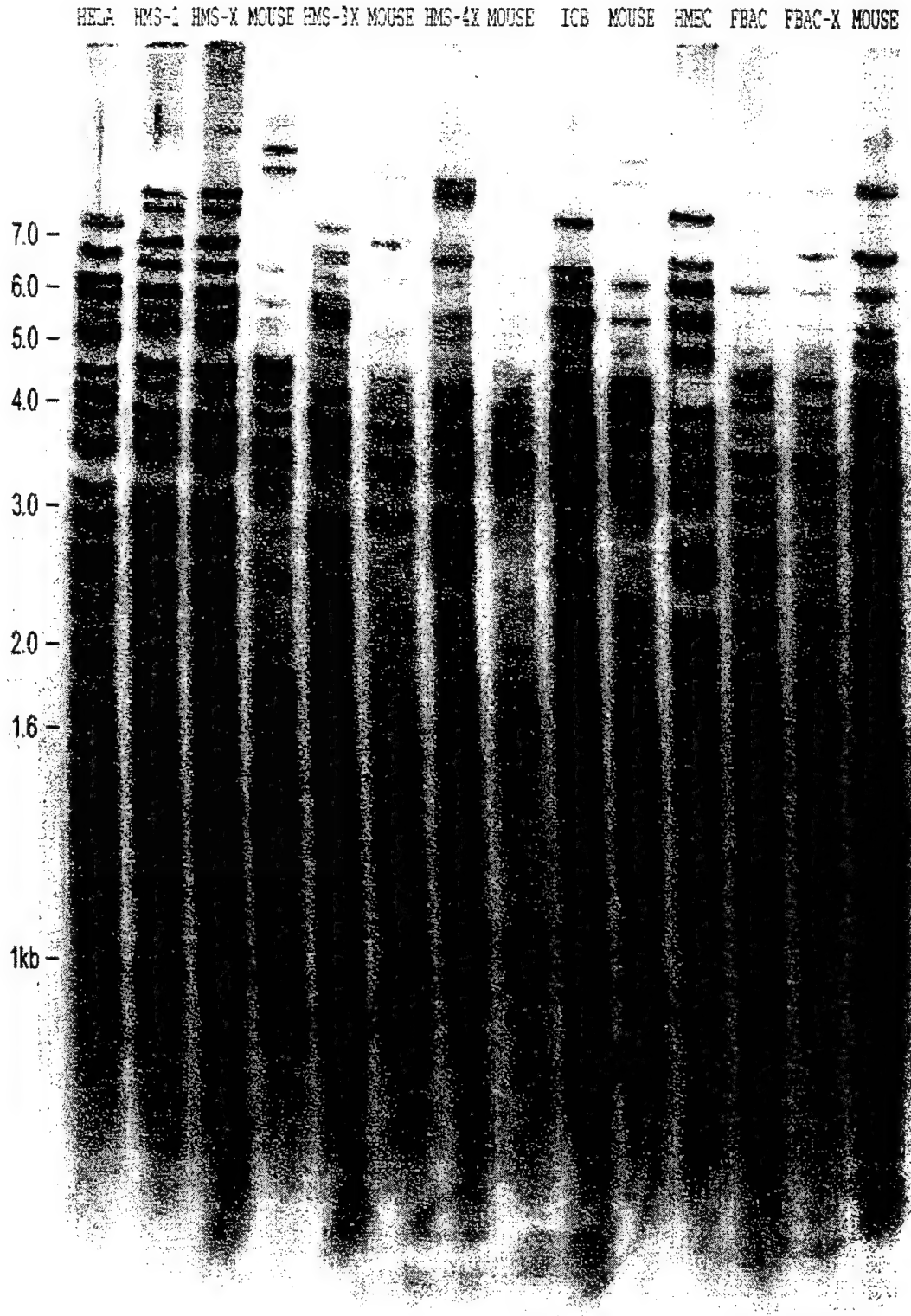

HELA
HMS-1
HMS-X MOUSE
HMS-3X MOUSE
HMS-4X MOUSE
IBC
MOUSE
HMEC
FBAC
FBAC-X MOUSE
7.0
6.0
5.0
4.0
3.0
2.0
1.6
1kb
HinfI

FIG. 5


HELA
HMS-1
HMS-X MOUSE
HMS-3X MOUSE
HMS-4X MOUSE
ICB
MOUSE
HMEC
FBAC
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1.6
1kb
HaeIII

FIG. 6

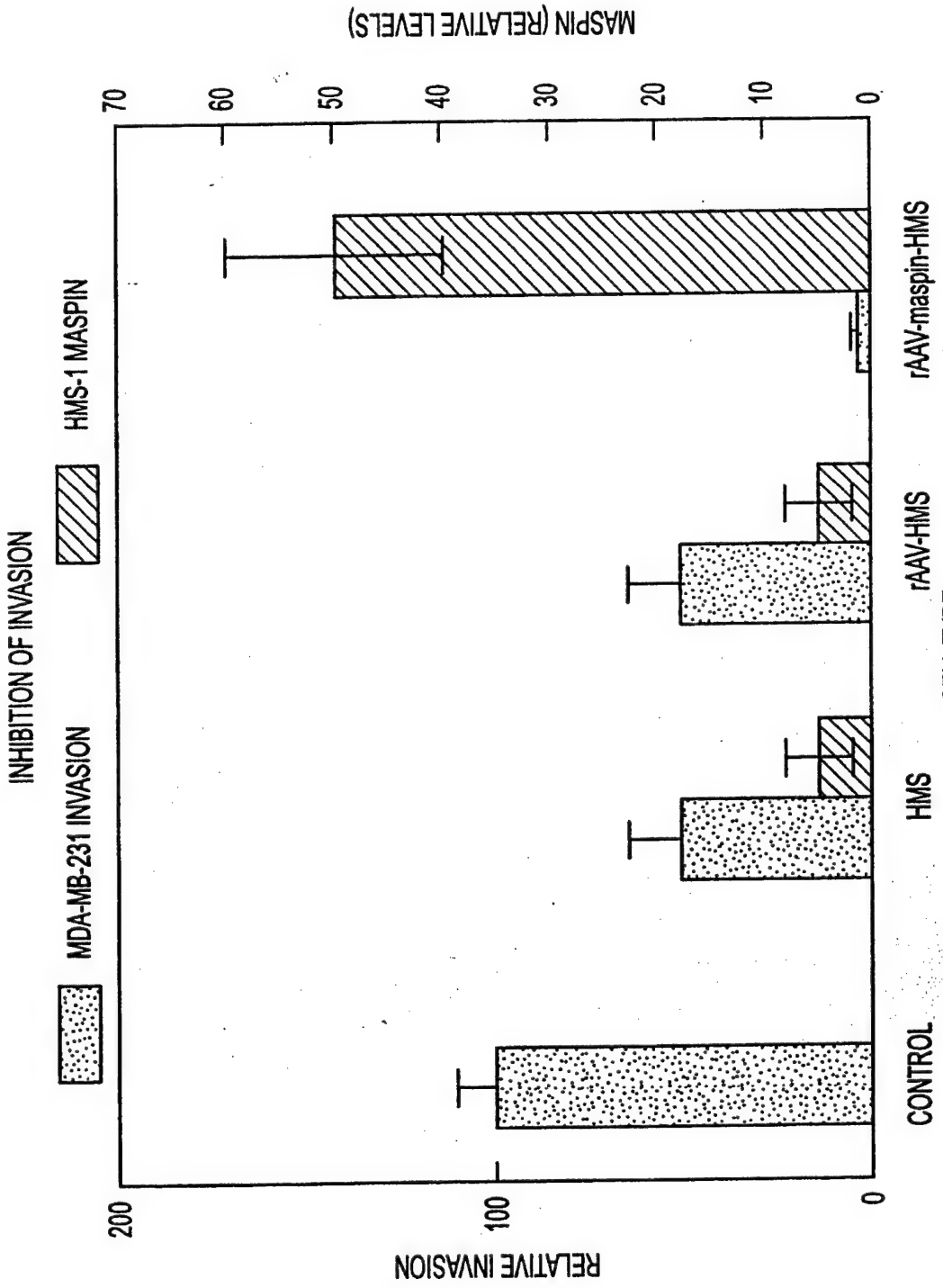
MASPIN (RELATIVE LEVELS)
70
60
50
40
30
20
10
0
INHIBITION OF INVASION
HMS-1 MASPIN
MDA-MB-231 INVASION
rAAV-maspin-HMS
rAAV-HMS
HMS
CONTROL
CELL TYPE
200
100
0
RELATIVE INVASION

FIG. 7

COMPOSITIONS AND METHODS FOR INTRADUCTAL GENE THERAPY

This application claims the benefit of U.S. provisional patent application Serial No. 60/116,470, filed Jan. 20, 1999. The entire contents of this provisional patent application is incorporated by reference into this application.

This invention was made with Government support under U.C. Biostar Biotechnology grant S98-42, grant No. BC990959, awarded by the Department of Defense Breast Cancer Research Program and grant No. DAMD 17-96-C6117, awarded by the Department of the Army, U.S. Army Medical Research Acquisition Activity. The government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to the use of cell specific vectors in the diagnosis as well as the prophylactic and therapeutic treatment of diseases of the breast, in particular cancer.

BACKGROUND OF THE INVENTION

Cancers of the breast are one of the leading causes of death among women, with the cumulative lifetime risk of a woman developing breast cancer estimated to be 1 in 9. Consequently, understanding the origins of these malignancies as well as the identification of new therapeutic modalities is of significant interest to health care professionals.

The mature human breast comprises from six to nine major ducts, which emanate from the nipple, serially branch into ducts and terminate in lobuloalveolar structures (Russo et al., Lab. Invest. 62(3): 244–278 (1990)). The branching network of ducts is composed of luminal epithelial cells in a supporting matrix of connective tissue and myoepithelial cells. Tissues removed from the human female breast during surgery and autopsy have been examined in numerous studies directed at the nature and site of origin of neoplastic growth. Subgross sampling and histological confirmation have enabled pathological characterization of entire breasts which shows that human breast cancer arise within the ductal system of the breast exclusively from luminal epithelial cells. Ductal origin is supported by the presence of more extensive epithelial proliferations, which are presumed to be preneoplastic, in surgically removed cancerous breasts as compared to nonmalignant breasts removed during autopsies.

With the significant cumulative lifetime risk of a woman developing breast cancer, there is an urgent need to develop both therapeutic methods of treatment that are more effective, less invasive and accompanied by fewer side effects as well as prophylactic methods of treatment that are more effective than increased and intensified physical monitoring and far less extreme than radical mastectomy. In spite of the recent discovery of the heritable breast cancer susceptibility loci including BRCA1 and other genes (see e.g. Miki et al., Science 266:66–71 (1994)), and the increasing ability of physicians to identify women with elevated breast cancer risk, prophylactic methods are still currently limited to physical monitoring and prophylactic mastectomy.

In view of the above, what is needed in the art is the identification of novel methods which aid in the prevention and treatment of cancers of the mammary gland. In this context, optimal methods are those which have a wide application both in the diagnosis of cancer, as well as the prophylactic and therapeutic treatment of cancer.

SUMMARY OF THE INVENTION

The present invention is based on the observations of the respective roles of the epithelial and myoepithelial cell lineages in the development of breast cancer and the fact that these cell types differentially express gene products that can be targeted by cell specific vectors. In this context, new strategies emerge which can be used as a means of breast carcinoma diagnosis, prophylaxis and treatment. A first strategy is to selectively target cells of the breast epithelium so that breast carcinoma does not develop. A second strategy is to target myoepithelial cells as a means of bolstering the myoepithelial defense so that even if DCIS develops, it will be confined to the ductal system indefinitely.

The present invention provides prophylactic and therapeutic methods of treating a disease of the ductal epithelium of the mammary gland, in particular cancer. The present invention further provides diagnostic methods of assessing the status of cancers of the mammary gland. In this context, the present invention provides methods directed to selectively transducing a cell in a ductal system in a mammary gland comprising the step of using ductal cannulation to contact the cell with a vector that selectively targets that cell. In an illustrative embodiment, the invention consists of a method of selectively transducing either a epithelial cell or a myoepithelial cell, by contacting the cell with a vector that selectively targets a CAR molecule that is expressed on the epithelial cell or a heparin sulfate proteoglycan molecule that is expressed on the myoepithelial cell.

In a specific embodiment of the invention, the vector is a replication defective adenovirus which targets a molecule expressed solely by a epithelial cell and subsequently induces cell death by delivery of an cytolysis inducing gene such as thymidine kinase or cytosine deaminase. In a more preferred embodiment, the vector is a replication-competent lytic adenovirus which targets a coxsackievirus and adenovirus receptor (CAR) molecule expressed by the epithellal cell and subsequently induces cell death by lysis. In a highly preferred embodiment, the replication-competent lytic adenovirus contains a cis element such as a lactoalbumin promoter and the MUCI promoter which stimulates adenovirus replication in the presence of a trans factor present in the epithelial cell and induces more effective lysis. The myoepithelial cells of the breast duct, which lack CAR expression (as shown, for example by RT-PCR), are completely resistant to adenovirus infection (transduction) and serve as a barrier to systemic infection.

In another specific embodiment of the invention, the vector is a recombinant adeno-associated virus which targets a molecule expressed by a myoepithelial cell and comprises a polynucleotide which encodes a polypeptide which inhibits the development of epithelial cell cancer. In a specific embodiment, the polypeptide of the recombinant adeno-associated virus inhibits angiogenesis or the proliferation, invasion or metastases of a epithelial cell. In a specific embodiment, the polypeptide is maspin, thrombospondin-1, TIMP-1, protease nexin-II, α-1 antitrypsin or soluble bFGF receptor. In a more preferred embodiment, the recombinant adeno-associated virus contains a cis element such as a lactoalbumin promoter and the MUCI promoter which stimulates recombinant adeno-associated virus replication in the presence of a trans factor present in the epithelial cell.

The present invention also provides a method of treating the ductal epithelium of a mammary gland prophylactically for cancer, which method comprises the step of contacting, by ductal cannulation, the ductal epithelium of the mammary gland with a tissue specific vector so as to inhibit the formation of cancer of ductal epithelial origin. In addition, the present invention provides combined therapeutic/prophylactic methods of treating the mammary gland therapeutically by surgery, radiation and/or chemotherapy and,

either concomitantly or subsequently, contacting the ductal epithelium of the mammary gland with a cell specific vector which specifically targets a epithellal or a myoepithelial cell.

The present invention also provides a method of determining the lineage of a cell in a ductal system in a mammary gland selected from the group consisting of a luminal epithelial cell and a myoepithelial cell, by using ductal cannulation to contact the cell with vector containing a reporter gene, wherein the vector selectively targets a molecule that is expressed on the epithelial cell or the myoepithelial cell.

In addition, the present invention provides compositions including recombinant adenovirus and recombinant adeno-associated virus vectors as well as myoepithelial cell lines and transplantable xenografts.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic which depicts the ductal-lobular unit of the breast and illustrates the possibility of gene therapy strategies targeting either epithelial cells and/or myoepithelial cells.

FIGS. **2**A is a photograph illustrating the feasibility of gaining access to the entire ductal system of the breast through nipple duct identification and cannulation.

FIG. **2**B, a photograph showing that when nipple ducts are individually cannulated, an injected dye reaches every ductal orifice.

FIG. **3**A is a photograph showing the absence of β-galactosidase expression in myoepithelial cells contacted with a β-galactosidase containing recombinant adenovirus (Ad2) to illustrate how the expression of CAR on the surface of epithelial cells in the ductal-lobular unit of the breast allows the selective targeting of the epithelial cells and how the absence of CAR on myoepithelial cells confers resistance of this cell to adenoviral infection.

FIG. **3**B is a photograph showing the intense β-galactosidase expression in epithelial cells contacted with a β-galactosidase containing recombinant adenovirus as an illustration of how the expression of CAR on the surface of epithelial cells in the ductal-lobular unit of the breast allows the selective targeting of epithelial cells.

FIG. **3**C is a photograph showing how CAR expression is completely absent in myoepithelial cells (lane under CAR) but present in ductal epithelial cells and carcinoma lines (other lanes).

FIG. **4**A is a photograph showing reporter gene expression in myoepithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene which illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells in the ductal-lobular unit of the breast allows the selective targeting of the myoepithelial cells.

FIG. **4**B is a photograph showing only background reporter gene expression in epithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene which illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells in the ductal-lobular unit of the breast allows the selective targeting of the myoepithelial cells.

FIG. **5** is a Southern blot of DNA cut with Hinf I and probed using the multi-locus Jefferys probe (33.6) showing the distinct and identifying pattern of a number of illustrative myoepithelial cell lines and xenografts disclosed herein (HMS-1, HMS-X, HMS-3, HMS-3X, HMS-4X). All of the myoepithelial cell lines and xenografts exhibited the same susceptibility to recombinant adeno-virus associated virus transfection and the same resistance to adenoviral transfection. Other myoepithelial cell lines and xenografts (HMS-5X, HMS-6X, HMS-5, HMS-6) not depicted exhibit the same properties.

FIG. **6** is a Southern blot of DNA cut with Hae III and probed using the multi-locus Jefferys probe (33.6) showing the distinct and identifying pattern of a number of illustrative myoepithelial cell lines and xenografts disclosed herein (HMS-1, HMS-X, HMS-3, HMS-3X, HMS-4X). All of the myoepithelial cell lines and xenografts exhibited the same susceptibility to recombinant adeno-virus associated virus transfection and the same resistance to adenoviral transfection. Other myoepithelial cell lines and xenografts (HMS-5X, HMS-6X, HMS-5, HMS-6) not depicted exhibit the same properties.

FIG. **7** is a bar graph comparing MDA-MB-231 breast carcinoma cell invasion in MATRIGEL matrix in the presence of no myoepithelial cells (control), HMS myoepithelial cells (HMS), rAAV transfected HMS myoepithelial cells (rAAV-HMS) and rAAV-recombinant maspin transfected HMS myoepithelial cells (rAAV-maspin-HMS). The results demonstrate that myoepithelial cells which overexpress maspin are highly effective at blocking carcinoma cell invasion in MATRIGEL matrix. Specifically, the effects of transfected myoepithelial clones expressing recombinant maspin in invasion inhibition assays show a 200% increase in inhibition of invasion.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "transduce" is used in its broadest sense and refers to a process wherein a vector gains entry in to a cell so that polynucleotides of the vector are delivered to the cell.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. eucaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Lining may be accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers maybe used in accordance with conventional practice.

"Polynucleotide" and "nucleic acid" refer to single- or double-stranded molecules which may be DNA, comprised of the nucleotide bases A, T, C and G, or RNA, comprised of the bases A, U (substitutes for T), C, and G. The polynucleotide may represent a coding strand or its complement. Polynucleotide molecules may be identical in sequence to the sequence which is naturally occurring or may include alternative codons which encode the same

amino acid as that which is found in the naturally occurring sequence (See, Lewin "Genes V" Oxford University Press Chapter 7, pp. 171–174 (1994)). Furthermore, polynucleotide molecules may include codons which represent conservative substitutions of amino acids as described. The polynucleotide may represent genomic DNA or cDNA.

"Polypeptide" refers to a molecule comprised of amino acids which correspond to those encoded by a polynucleotide sequence which is naturally occurring. The polypeptide may include conservative substitutions where the naturally occurring amino acid is replaced by one having similar properties, where such conservative substitutions do not alter the function of the polypeptide (See, Lewin "Genes V" Oxford UniversityPress Chapter 1, pp.: 9–13 (1994)).

The gene therapy strategies provided herein are predicated on either selectively destroying the breast epithelial cell (where cancers arise) and/or enhancing the functions of the myoepithelial cell (which are natural cancer suppressors). The present invention utilizes observations of the different of the ductal epithelial and ductal myoepithelial cell lineages in the development of breast cancer and the fact that these cell types differentially express gene products that can be targeted by cell specific vectors. In this context, new strategies emerge which can be used as a means of breast carcinoma diagnosis, prophylaxis and treatment. A first strategy is to selectively transduce luminal epithelium cells so that breast carcinoma does not develop. A second strategy is to selectively transduce myoepithelial cells as a means of bolstering the myoepithelial defense so that even if DCIS develops, it will be confined to the ductal system indefinitely.

The present invention takes advantage of observations that early breast cancer can be thought of as a disease of the ductal system which arises from ductal epithelium and that human breast cancer arises within the ductal system of the breast exclusively from epithelial cells that are located within the breast duct. Throughout this application, the epithelial cells from the breast duct are referred to by a variety of art accepted designations such as ductal epithelial cells, acinar epithelial cells, luminal epithelial cells etc. (see e.g. Virchows et al., Arch. 391(1): 45–51 (1981); Cristov et al., Am. J. Pathol. 138(6): 1371–7 (1991); Lochter, Biochem. Cell Biol. 76(6): 997–1008 (1998); Lakhani et al., J. Pathol. 189(4): 496–503 (1999)) The various designations are used for purposes of clarity in the context in which they are discussed and all refer to epithelial cells which can be selectively transduced by vectors which target CAR expressed by these cells. Preferably, such cells reside within the breast duct system. When cancer is confined by this system it is termed ductal carcinoma in situ or DCIS. Recent evidence suggests that the determinants of the progression of DCIS to invasive cancer are epigenetic rather than genetic and consist of strong paracrine regulation by neighboring myoepithelial cells (T. Kuukasjarvi et al. (1997) Am. J. Pathol., 150:1465–1471). Myoepithelial cells are cells which surround this ductal system and keep the developing cancer termed ductal carcinoma in situ (DCIS) confined. Because of their close proximity, myoepithelial cells would be anticipated to exert important paracrine influences on DCIS progression. Myoepithelial cells accomplish this by their production and secretion of a number of proteinase inhibitors.

Myoepithelial cells of the breast differ from luminal ductal and acinar epithelial cells in many ways: they lack expression of the common hormonal receptor, ER-I, and its responsive genes like PR; they lie next to the basement membrane and contribute to the synthesis of that structure; they rarely transform or proliferate and when they do give rise to only low grade benign neoplasms M. D. Sternlicht and S. H. Barsky (1997) Medical Hypotheses, 48:37–46). Myoepithelial cells are ubiquitously present in normal ducts, benign proliferations such as adenosis and precancerous proliferations, e.g. DCIS. These cumulative studies suggest that DCIS is cancer of the breast in the true genetic, biological and clinical sense of the word but is limited within the confines of the ductal system by myoepithelial cells. Both epithelial cells and myoepithelial cells can then be targets of intraductal gene therapy provided that specific vectors can be identified which will selectively target each cell type.

The findings disclosed herein allow specific gene therapy approaches to carry out the strategies discussed above. A first finding is that it is possible to gain access to the entire ductal system of the breast through nipple duct identification and cannulation. A second finding is that primary mammary ductal epithelial cells express the coxsackievirus-Ad receptor (CAR, see e.g. J. Bergelson et al. (1997) Science 275:1320–1323) and are easily transduced with vectors that gain entry to the cell via CAR mediated uptake (such as certain adenovirus groups) whereas myoepithelial cells lack CAR and are completely resistant to transduction by vectors which target this molecule. As illustrated in Table I below, a third finding is that myoepithelial cells, do not express CAR and instead express cell surface heparin sulfate proteoglycan (see e.g. Summerford et al., J. Virol. 72(2), 1438–1445 (1998)) and are easily transduced with vectors that gain entry to the cell via heparin sulfate proteoglycan mediated uptake such as rAAV) whereas primary ductal epithelial cells do not express this proteoglycan and are resistant to transduction by vectors which target this molecule. These findings are supported by a number of experiments with established myoepithelial xenografts and cell lines (both immortalized and in limited short term passage), which further demonstrate the selectiveness of the rAAV for the myoepithelial target.

Table 1 depicts high cell surface heparin sulfate proteoglycan content of myoepithelial cells (HMS-1)

	Uronic Acid[b]		Glycosaminoglycan Content (%)				
	(dry wt)	(DNA)	CS	HA	HS	DS	KS
Normal salivary gland	1.83	0.031	4	56	24	16	0
Basal cell adenoca (HMS-1)	0.63	0.008	32	12	24	0	32
Basal cell adenoca (HMS-X)	10.45	0.530	78	12	8	2	0
Adenoidcystic Ca (HMS-3X)	8.96	0.298	77	11	9	3	0
Pleomorphic adenoma	26.49	3.718	89	7	0	4	0
Squamous cell Ca (A253)	0.77	0.009	19	59	14	8	0
Squamous cell Ca (A253-X)	3.93	0.094	30	27	31	12	0
Murine EHS tumor	7.62	0.356	0	6	94	0	0
Cartilage	ND	ND	61	15	3	21	0

All data represent mean values obtained from two or more determinations from the same sample.
Uronic acid was measured in micrograms per milligram of tissue (dry weight) and micrograms per microgram of DNA. CS, chondroitin sulfate: HA, hyaluronic acid; HS, heparin sulfate; DS, dermatan sulfate; KS, keratin sulfate; not determined.

The findings disclosed herein illustrate the feasibility of, using an intraductal approach and different cell specific viral vectors such as rAd, selectively targeting and destroying the

breast epithelium in vivo while sparing the underlying myoepithelium Further, these findings illustrate the feasibility of, using an intraductal approach and different viral vectors such as rAAV, selectively targeting the myoepithelium in vivo with a vector containing a molecule capable of inhibiting epithelial luminal cell growth and invasion as a means of bolstering the suppressive effects of these cells. In addition, these findings allow for the utilization of replication-defective rAd and rAAV containing reporter genes in diagnostic studies which evaluate the expansion of a specific cell type. While various embodiments of the methods disclosed herein can be used to treat any exocrine gland, they are particularly useful in the treatment of the mammary gland.

The methods disclosed herein overcome a number of limitations of methods known in the art. Specifically, while a number of vectors (e.g. vaccinia, sindbis and certain adenoviral and retroviral vectors) exhibit a wide tissue tropism and are used to transduce a variety cell lineages including epithelial and myoepithelial cells, the broad specificity of these vectors can also limit their usefulness. In particular, while vectors having a wide tissue tropism are favored in certain contexts, this property is disadvantageous in situations where it is desirable to selectively transduce a specific cell lineage that is present in a mixed population of different cell lineages. For example, practitioners using vectors having a wide tissue tropism in vivo may be forced to use them only in contexts where a host's rapid immunological response will prevent them from escaping the milieu in to which they are introduced. The vectors and methods disclosed herein overcome such problems by allowing the artisan to selectively transduce a specific lineage (such as epithelial or myoepithelial cells) that is present within a mixed population of cells of different lineages. Specifically, methods utilizing vectors which selectively transduce a specific lineage restrict the ability of such vectors to gain entry into proximal cells of different lineages (which are not transduced by the vector), and consequently inhibit such vectors from escaping the milieu in to which they are introduced.

Vectors which Selectively Transduce Epithelial Cells

A significant feature of the methods of the invention disclosed herein is the identification and utilization cell specific vectors which have the ability to selectively transduce epithelial cells and not transduce myoepithelial cells. Specifically, as these vectors gain entry into target cells via a molecule which is expressed on epithelial but not myoepithelial cells, they can be used to selectively transduce epithelial cells within a mixed population of epithelial and myoepithelial cells.

A variety different vectors which gain entry into cells through the CAR molecule can be used to selectively transduce epithelial cells. Strains of the coxsackie B viruses (such as coxsackie B3 and B4) for example enter cells via CAR mediated uptake (see e.g. Bergelson et al., J. Virol. 72(1), 415–419 (1998)). Therefore, coxsackie virus vectors can be used to selectively transduce an epithelial cell in a ductal system in a mammary gland that expresses CAR molecules. In addition, certain subgroups of adenovirus also gain entry into cells via CAR. In particular, adenovirus serotypes from subgroups A (e.g. Ad12), C (e.g. Ad1, Ad2, Ad5 and Ad6), D (e.g. Ad9, Ad15, Ad30 and Ad37), E (e.g. Ad4), and F (e.g. Ad40 and Ad41) all use CAR as a cellular fiber receptor (see e.g. Roelvink et al., J. Virol. 72(10): 7909–7915 (1998)). It is however important to note that adenoviruses can target different molecules expressed by target cells and that not all adenovirus strains can be used to selectively transduce CAR expressing epithelial cells (see e.g. Nalbantoglu et al., Hum. Gene Ther. 10(6): 1009–1019 (1999). For example, subgroup B adenoviruses (e.g. Ad3, Ad7 and Ad35) appear to interact with target cells through a different cellular receptor than CAR and may not function in the disclosed methods (see e.g. Stevenson et al., J. Virol. 69(5): 2850–2857 (1995)). Therefore it is important to establish that any vector (adenovirus or otherwise) used in the disclosed methods can selectively transduce epithellal cells while not transducing proximal myoepithelial cells.

In addition to vectors having a natural tropism for CAR, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule on a cell such as CAR. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Pat. No. 5,834,589 incorporated herein by reference). Because the CAR binding adenoviral fiber protein residues have been identified (see e.g. Santis et al., J. Gen. Virol. 80(Pt 6): 1519–1527 (1999)) and because the adenovirus binding activity of CAR has been localized to the amino-terminal IgV-related domain of this molecule, one skilled in the art generate target vectors specific for this binding domain.

As noted above, certain viral vectors may be able to gain entry in to cells through multiple receptors expressed on the surface of a cell. For example, certain adenoviral subtypes may be able to gain entry in to cells via the MHC class I α2 domain or members of the β2 integrin family (see e.g. Davison et al., J. Virol. 73(5): 4513–4517 (1999) and Huang et al. J. Virol. 70(7): 4502–4508 (1996)). Therefore, it is important to establish that any vector (adenovirus or otherwise) used to selectively transduce epithelial cells will not transduce proximal myoepithelial cells. In particular, as certain vectors may be able to gain entry into a target cell by more than one receptor, it is prudent to assess the cellular specificity of any candidate vectors. Methods for assessing the specificity of a candidate vector are well known in the art and consist merely of contacting a target cell of interest with a candidate vector and observing if transduction occurs. In this context, FIG. 3 provides an illustrative example of an assessment of the adenoviral vector used in the disclosed examples. In particular, FIG. 3A shows the absence of β-galactosidase expression in myoepithelial cells contacted with a β-galactosidase containing recombinant adenovirus to illustrate how the expression of CAR on the surface of epithelial cells allows the selective targeting of the epithelial cells and how the absence of CAR on myoepithelial cells confers resistance of this cell to adenoviral infection. In contrast, FIG. 3B shows the intense β-galactosidase expression in epithelial cells contacted with a β-galactosidase containing recombinant adenovirus as an illustration of how the expression of CAR on the surface of epithelial cells allows the selective transduction of epithelial cells.

Vectors which Selectively Transduce Myoepithelial Cells

A significant feature of the methods of the invention disclosed herein is the identification and utilization of cell specific vectors which have the ability to selectively transduce myoepithelial cells and not transduce epithelial cells. Specifically, as these vectors gain entry into target cells via a molecule which is expressed on myoepithelial but not epithelial cells, they can be used to selectively transduce myoepithelial cells within a mixed population of myoepithelial and epithelial cells.

Different vectors which gain entry into cells through the heparin sulfate proteoglycan molecule can be used to selec-

tively transduce myoepithelial cells. The human parvovirus, Adeno-associated virus-2 (AAV) for example can enter cells via heparin sulfate proteoglycan mediated uptake (see e.g. C. Summerford and R. J. Samulski, J. Virology 72:1438–1445)). In addition, herpesviruses are believed to target cells through their hepatin sulfate proteoglycan molecules (see e.g. Zhu et al., P.N.A.S. 92(8): 3546–3550 (1995)). It is however important to note that viral vectors can attach different molecules expressed by target cells and/or may utilize co-receptors to gain entry in to a cell (see e.g. Summerford et al., Nat. Med. 5(1): 78–82 (1999) and Qing et al., Nat. Med. 5(1): 71–11 (1999)). Therefore it is important to establish that any vector (adeno-associated virus-2 or otherwise) used in the disclosed methods can selectively transduce myoepithelial cells while not transducing proximal epithelial cells.

In addition to vectors having a natural tropism for heparin sulfate proteoglycan, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule on a cell such as heparin sulfate proteoglycan. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Pat. No. 5,834,589 incorporated herein by reference).

As noted above, certain viral vectors may be able to gain entry in to cells through multiple receptors expressed on the surface of a cell. For example, adeno-associated virus 2 may be able to gain entry in to cells via the human fibroblast growth factor receptor 1 or αVβ integrin (see e.g. see e.g. Summerford et al., Nat. Med. 5(1): 78–82 (1999) and Qing et al., Nat. Med. 5(1): 71–11 (1999)). Therefore, it is important to establish that any vector (adeno-associated virus 2 or otherwise) used to selectively transduce myoepithelial cells will not transduce proximal epithelial cells. In particular, as certain vectors may be able to gain entry into a target cell by more than one receptor, it is prudent to assess the cellular specificity of any candidate vectors. Methods for assessing the specificity of a candidate vector are well known in the art and consist merely of contacting a target cell of interest with a candidate vector and observing if transduction occurs. In this context, FIG. **4** provides an illustrative example of an assessment of the Adeno-associated virus-2 used in the disclosed examples. In particular, FIG. **4**A shows reporter gene expression in myoepithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene and illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells allows the selective targeting of the myoepithelial cells. In comparison, FIG. **4**B shows only background reporter gene expression in epithelial cells contacted with a recombinant adeno-associated virus containing a human green fluorescence reporter gene and illustrates how the expression of heparin sulfate proteoglycan on the surface of myoepithelial cells allows the selective transduction of the myoepithelial cells.

Generation and Manipulation of Vectors of the Invention

Methods for generating and manipulating vectors such as the recombinant adeno-associated virus and adenovirus vectors disclosed herein are well known in the art, for example in U.S. Pat. Nos. 5,681,731, 5,585,362, 5,756,283 and 5,843,742 which are incorporated herein by reference. As discussed below, a variety of art accepted techniques may be utilized in the practice of embodiments of the invention disclosed herein. While the vectors of the invention may be know in the art such as replication-competent lytic adenoviruses, the vectors can also be manipulated to facilitate their use in the methods disclosed herein. For example, a nucleic acid (eg., cDNA or genomic DNA) containing a regulatory region such as a promoter or an enhancer may be inserted into a cell specific vector for cloning (amplification of the DNA) or for expression. Alternatively, a nucleic acid encoding a gene of interest such as a suicide gene or tumor suppressive gene maybe inserted into a cell specific vector for expression in a target cell.

The nucleic acid sequence of interest may be inserted into a vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

Both expression and cloning vectors can contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of mammalian, bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2: plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up a nucleic acid of interest, such as Neomycin, DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., *Proc. Natl. Acad. Sci. USA,* 77:4216 (1980). A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp^7 (Stinchcomb et al., *Nature,* 282:39 (1979); Kingsman et al., *Gene,* 7:141 (1979); Tschemper et al., *Gene,* 10:157 (1980)). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics,* 85:12 (1977)).

Expression and cloning vectors can contain a promoter operably linked to the nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang et al., *Nature,* 275:615 (1978); Goeddel et al., *Nature,* 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.,* 8:4057 (1980); EP 36,776), and hybrid promoters such as the tac promoter (deBoer et al., *Proc. Natl. Acad. Sci. USA,* 80.21–25 (1983)). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding a nucleic acid of interest.

Transcription from vectors in mammalian host cells can be controlled, for example, by promoters obtained from the

genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilioma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalin promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems. In addition, a polynucleotide of interest can be under the transcriptional regulation of an inducible promoter such as the Tet promoter. Alternatively, it can be under the control of an epithelial tissue-specific or cell-specific promoter. Epithelial cell-specific promoters, such as whey acidic protein (wap), can be used to target expression of a given gene, e.g., a suicide gene, in ductal epithelial cells. Use can also be made of promoters which control wild-type tumor suppressor genes, such as Maspin, p53 or Mcs-1 (rat), homeobox genes which are expressed in normal cells but not in cancerous cells.

Transcription of a DNA encoding a polynucleotide of interest by mammalian cells may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, "-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eucaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100–270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the coding sequence, but is preferably located at a site 5' from the promoter.

Methods for Selectively Transducing Epithelial and Myoepithelial Cells

The methods of the invention disclosed herein allow the selective transduction of both ductal epithelial and myoepithelial cells. An illustrative embodiment of the invention is a method of selectively transducing a cell within a mixed population of ductal epithelial and myoepithelial cells, comprising the step of contacting the cell with a cell specific vector that transduces the cell through a molecule that is expressed only on the ductal epithelial cell or, alternatively, through a different molecule that is expressed only on the myoepithelial cell. In a preferred embodiment of this invention, the mixed population of ductal epithelial and myoepithelial cells is within a ductal system of a mammary gland and the cells to be transduced are contacted with the vector by ductal cannulation.

In a specific embodiment of this invention, cell is a ductal epithelial cell that is transduced with a vector (such as a replication competent adenovirus) that induces cell death. In a related embodiment, the vector can contain a suicide gene that can induce cell death such as thymidine liase or cytosine deaminase. In another specific embodiment of the invention, the vector contains a as element which stimulates viral replication or specific gene expression in the presence of a trans factor present in the ductal epithelial cell. For example, the cis element could be one of the large number of known regulatory sequences such as the lactoalbumin promoter and the MUCI promoter.

In yet another preferred embodiment of this invention, the cell is a myoepithelial cell that is transduced with a vector (such as a recombinant adeno-associated virus) that comprises a gene encoding a polypeptide which inhibits the development of cancer. In a specific embodiment of this invention, the vector contains a cis element contains a cis element which modulates the expression of the gene in the presence of a trans factor present in the myoepithelial cell. In preferred embodiments of this method, the polypeptide inhibits the proliferation of a ductal epithelial cell, the invasion of a ductal epithelial cell, endothelial migration, angiogenesis or increases the production of nitric oxide by the myoepithelial cell. Illustrative polypeptides include maspin, thrombospondin-1, TIMP-1, protease nexin-II, α-1 antitrypsin and soluble bFGF receptor. Preferably the polypeptide induces death (for example by apoptosis) of a ductal epithelial cell.

In a related embodiment, the invention consists of a method of selectively transducing a cell in a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a myoepithelial cell, comprising the step of contacting, by ductal cannulation, the cell with a vector that selectively targets a CAR molecule that is expressed on the ductal epithelial cell or a heparin sulfate proteoglycan molecule that is expressed on the myoepithelial cell. In yet another related embodiment, the invention consists of a method of selectively transducing a ductal epithelial cell in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a CAR molecule expressed by the cell. In yet another related embodiment, the invention consists of a method of selectively transducing a myoepithelial cell in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a heparin sulfate proteoglycan molecule expressed by the cell.

In a related embodiment, the invention is a method of transducing ductal epithelial cells within a mixed population of ductal epithelial and myoepithelial cells, while not transducing proximal myoepithelial cells, by contacting the ductal epithelial cells with a vector that gains entry into ductal epithelial cells via a CAR molecule expressed by the ductal epithelial cells. In yet another related embodiment, the invention consists of a method of transducing ductal epithelial cells within a mixed population of ductal epithelial and myoepithelial cells, wherein the myoepithelial cells are not transduced, by contacting the ductal epithelial cells with a vector that gains entry into the ductal epithelial cells via a CAR molecule expressed by the ductal epithelial cells but not expressed by the myoepithelial cells.

In yet another related embodiment, the invention consists of a method of transducing myoepithelial cells within a mixed population of ductal epithelial and myoepithelial cells, while not transducing proximal ductal epithelial cells, by contacting the myoepithelial cells with a vector that gains entry into myoepithelial cells via a heparin sulfate proteoglycan molecule expressed by the myoepithelial cells. In yet another related embodiment, the invention consists of a method of transducing myoepithelial cells within a mixed population of ductal epithelial and myoepithelial cells, wherein the ductal epithelial cells are not transduced, by contacting the myoepithelial cells with a vector that gains entry into the myoepithelial cells via a heparin sulfate proteoglycan molecule expressed by the myoepithelial cells but not expressed by the ductal epithelial cells.

Prophylactic Methods for Treating a Breast Duct

The prophylactic methods of the present invention involve methods of selectively treating a cell in the duct of a breast prophylactically for a disease that affects the ductal epithelium of the breast such as cancer. One embodiment of these methods consists of selectively transducing either a luminal epithellal cell, a myoepithelial cell (or both a luminal epithelial cell and a myoepithelial cell) within the duct of a breast, by using ductal cannulation to contacting the cell of interest with a vector that selectively targets a molecule that is expressed on either the luminal epithelial cell or the myoepithelial cell. Depending upon which cell is contacted, the vectors used in these methods are then used to effect a desired biological activity such as cytolysis or the expression of a soluble effector molecule.

In an exemplary embodiment, the method comprises contacting, preferably by ductal cannulation, a myoepithelial or epithelial cell with a cell specific vector that either suppresses the growth of cancerous cells or effects the destruction of all or less than all of the ductal epithelium so as to inhibit the formation of cancer of ductal epithelial origin. In another illustrative embodiment, the invention consists of a method of treating the ductal epithelium of a mammary gland for cancer of ductal epithelial cell origin, comprising the step of contacting, by ductal cannulation, a cell in a ductal system in a mammary gland selected from the group consisting of a ductal epithelial cell and a myoepithelial cell, with a vector that enters the cell through a CAR molecule expressed by the ductal epithelial cell or a heparin sulfate proteoglycan molecule expressed by the myoepithelial cell, wherein the expression of the polynucleotides of the vector so transduced inhibits the formation of cancer of ductal epithelial cell origin.

In a specific embodiment of the prophylactic methods of the present invention, epithelial cells of the mammary gland are treated prophylactically with a cytotoxic cell specific vector so as to inhibit the formation of cancer of ductal epithelial origin. In this context, only those cytolytic viruses which exhibit the proper cell specificity (i.e. those which selectively transduce epithelial cells via the CAR molecule expressed by these cells) can be used in the methods of the invention. In another specific embodiment of the prophylactic methods of the present invention, myoepithelial cells of the mammary gland are treated prophylactically with a cell specific vector comprising a polynucleotide encoding a suppressor molecule so as to inhibit the formation of cancer of ductal epithelial origin.

The prophylactic methods of treating a mammary gland described herein are particularly useful in treating a mammary gland in a mammal at risk for developing breast cancer. The mammary gland can be characterized as one that has never had a tumor, one that had a tumor previously but the tumor is no longer detectable due to other prior therapeutic treatment, or one that has an incipient or occult tumor, preneoplasia or ductal hyperplasia. Normally, hyperplasias and incipient and occult tumors are not detectable by means of physical examination or radiology. Accordingly, the prophylactic method will find use in cases where there is reason to take some prophylactic measures, such as when there are known inherited factors predisposing to cancers, where there are suspicious lesions present in a breast with the potential for developing into a malignancy, where there has been exposure to carcinogenic agents in the environment, where age predisposes to a cancer, where cancer of another gland, e.g., the mammary gland of the contralateral breast, suggests a propensity for developing cancer, or where there is a fear or suspicion of metastasis.

The methods of the present invention can also be combined with other methods of prophylactic and therapeutic treatment in addition to those cited herein, such as methods that target destruction of cancer cells, e.g., by targeting of cell-surface markers, receptor ligands, e.g., ligands to gastrin-releasing peptide-like receptors, tumor-associated antigens, e.g., the 57 kD cytokeratin or the antigen recognized by the monoclonal antibody GB24, the extracellular matrix glycoprotein tenascin, antisense oncogenes such as c-fos, homeobox genes that are expressed in cancer cells but not normal cells, tumor-infiltrating lymphocytes that express cytokines, RGD-containing peptides and proteins, which are administered following surgery, lipophilic drug-containing liposomes to which are covalently conjugated monoclonal antibodies for targeting to cancer cells, low fat diet, moderate physical exercise and hormonal modulation.

Introduction of Vectors via Ductal Cannulation

The ductal epithelium is preferably contacted with the agent by introduction of the agent through the duct of the exocrine ductal epithelium, such as by ductal cannulation. In the mammary gland, there are 6–9 major ducts that emanate from the nipple and serially branch into other ducts, terminating in lobulo-alveolar structures (Russo et al. (1990), supra). Accordingly, in some circumstances, such as those in which even more localized treatment is necessary or desired, for example, by the choice of anti-cancer agent, it may be preferable to contact the ductal epithelium of the exocrine gland through one of these major ducts connecting to a lobulo-alveolar structure. In this regard, ductal cannulation enables intratumoral injection.

Methods of ductal cannulation are known in the art (see e.g. S. M. Love and S. H. Barsky (1996) The Lancet, 348:997–999; Makita et al., Breast Cancer Res. Treat. 18: 179–188 (1991) and Okazaki et al., Jpn J Clin Oncol 21: 188–193 (1991)). Briefly, a breast can prepared and draped, with the breast-duct orifices identified with magnifying loupes or the methods described below. One or more ducts can then be cannulated with cannula known in the art such as a rigid metal duct-probe (6 FR Taber-Rothschild Galactography Kit, Manan Medical Products Inc., Northbrook, Ill.).

In order to facilitate the location individual orifices in a nipple of a breast, one can employ methods for transiently marking and locating individual orifices in a nipple of a breast as further described in U.S. Ser. No. 09/153,564, incorporated herein by reference. Moreover, methods for gaining access and evaluating cells of a breast duct as further described in U.S. Ser. Nos. 09/067,661 and 09/301,058, incorporated herein by reference. For example, one can introduce a detectable substance, such as a labeling reagent, dye, or the like, to the nipple so that the substance localizes and/or accumulates at or near the orifice to permit visual, automated, or other detection. Alternatively, one can utilize other stimuli for inducing a response, change, or reaction at or near a location of the orifice in the nipple. For example, it may be possible to illuminate the nipple with certain light or other energies which help distinguish between the orifice and other tissue surfaces. It may also be possible to introduce chemical reagents which react with ductal secretions at the orifice to enhance visibility e.g. to produce a visible or otherwise detectable reaction product.

Methods Targeting Epithelial Cells

In a specific embodiment of the prophylactic methods of the present invention, luminal epithelial cells in a mammary gland are treated prophylactically for cancer so as to inhibit the formation of cancer of ductal epithelial origin. The method comprises contacting, preferably by ductal cannulation, a luminal epithelial cell in the mammary gland with a vector that selectively targets the luminal epithelial cell. In a preferred embodiment, the vector is a replication competent adenovirus which targets a CAR molecule expressed by the luminal epithelial cell and subsequently induces cell death by lysis. In another embodiment, the vector contains a suicide gene such as thymidine kinase or cytosine deaminase and induces cell death via apoptosis. In a more preferred embodiment, the replication-competent lytic adenovirus contains a cis element such as a lactoalbumin promoter and the MUCI promoter which stimulates adenovirus replication in the presence of a trans factor present in the epithelial cell which enhances viral replication, lysis and cell death. With respect to the replication competent adenovirus strategy, the resistance of myoepithelial cells to adenovirus infection inhibits systemic infection by adenovirus and limits the gene and/or viral therapy to the ductal system of the breast.

Other vectors are contemplated in addition the adenovirus viral vectors described herein. For example, coxsackie viruses also target cells through the CAR molecule (see e.g. Bergelson et al., Science 275(5304): 1320–1323 (1997). Therefore, coxsackie virus vectors can also be used to

selectively transduce a cell in a ductal system in a mammary gland that expresses CAR molecules. Moreover, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule, such as CAR, on a cell. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Pat. No. 5,834,589).

As discussed above, a cell specific vector such as the adenoviral vectors disclosed herein can further comprise a suicide gene to enhance the destruction of epithelial cells in the breast. For example, a vector comprising a suicide gene, upon transformation of a epithelial cell and expression therein, renders the transformed cell sensitive to the epithelium-destroying agent, increases the sensitivity of the transformed cell to the agent, converts the agent from a prodrug to an active drug, activates the conversion of the agent from a prodrug to an active drug, enhances the effect of the agent or, itself, produces a protein that is cytotoxic. A preferred suicide gene for use in the present inventive methods is thymidine kinase, such as is found in the Herpes simplex virus, which phosphorylates nucleoside analogues including gancyclovir, which, in turn, inhibits DNA replication. Another example of a suicide gene is cytosine deaminase, which is used in conjunction with 5-fluorocycosine. If the vector comprising the suicide gene is administered locally to the ducts, the cytotoxic agent or precursor can be administered systemically, since only transfected cells will be affected. In this regard, the bystander effect, i.e., the death of neighboring uninfected cells, presumably due to transfer of toxic byproducts through gap junctions between cells in the same compartment, obviates the need for every cell in the ductal epithelium, which is to be destroyed, to be infected. However, sufficient time must be allowed between contacting the epithelial cell with the suicide gene and the prodrug, for example, to achieve efficient killing of the breast epithelial cells.

A cell specific vector comprising an apoptosis-inducing gene also can be used as an agent that destroys a epithelial cell of a breast (Vaux, Cell 76:777–779 (1994)). Examples of apoptosis-inducing genes include ced genes, myc genes (overexpressed), the bclxs gene, the bax gene, and the bak gene. The apoptosis-inducing gene causes death of transfected cells, i.e., by inducing programmed cell death. For example, the bclxs gene, bax gene, or bak gene can be used to inhibit bcl-2 or bcl-X(L), leading to apoptosis. Where necessary, a vector comprising an apoptosis-inducing gene can be used in combination with an agent that inactivates apoptosis inhibitors such as bcl-z, p35, IAP, NAIP, DAD1 and A20 proteins.

Suicide and apoptosis genes can be administered by way of a cell specific vector, such as the adenoviral vectors described in the Examples below. Adenoviral vectors are favored because they enable the generation of high titer recombinant viruses and the efficient transduction of post-mitotic cells because adenoviral DNA exists as an episome in the nucleus (Verma, Molecular Medicine 1:2–3 (1994)).

Methods Targeting Myoepithehal Cells

In another embodiment of the prophylactic methods of the present invention, the myoepithelium of a mammary gland can be treated prophylactically for cancer so as to inhibit the formation of cancer of ductal epithelial origin. The method comprises contacting, preferably by ductal cannulation, the myoepithelium of the mammary gland with a vector comprising a molecule which can inhibit the formation of cancer of ductal epithelial origin, for example, maspin. In a preferred embodiment, the vector is a recombinant adeno-associated virus which targets a heparin sulfate proteoglycan molecule expressed by the myoepithelial cell and comprises a polypeptide which inhibits the development of epithelial cell cancer. In a specific embodiment, the polypeptide of the recombinant adeno-associated virus inhibits angiogenesis or the proliferation, invasion or metastases of a luminal epithelial cell. In a specific embodiment, the polypeptide is maspin, thrombospondin-1, TIMP-1, protease nexin-II, α-1 antitrypsin or soluble bFGF receptor.

Other vectors are contemplated in addition the adeno-associated viral vectors described herein. For example, herpesviruses are believed to target cells through their heparin sulfate proteoglycan molecules (see e.g. Zhu et al., P.N.A.S. 92(8): 3546–3550 (1995)). Therefore, herpesvirus vectors such as those known in the art (see e.g. Levatte et al., Neuroscience 86(4): 1321–1336 (1998)), can also be used to selectively transduce a cell in a ductal system in a mammary gland that expresses heparin sulfate proteoglycan molecules. Moreover, it is known in the art that a wide variety of vectors may be constructed to target a specific molecule, such as heparin sulfate proteoglycan, on a cell. In particular, target cell specificity of delivery vectors can be provided by incorporation of a target cell specific binding domain by the use of any binding domain, which binds specifically to a binding site on the target cell (see e.g. U.S. Pat. No. 5,834,589).

Therapeutic Methods for Treating a Breast Duct

Embodiments of therapeutic methods of the present invention are related to and can parallel the prophylactic methods described above and include treating the ductal epithelium of a breast therapeutically for a disease that affects the ductal epithelium In one embodiment of the therapeutic methods of the present invention, the ductal epithelium of a mammary gland is treated therapeutically for cancer so as to destroy cancerous and noncancerous epithelial cells of the ductal epithelium and inhibit the spread of cancer. In an exemplary embodiment, the method comprises contacting, preferably by ductal cannulation, a myoepithelial or epithelial cell with a cell specific vector that can either effect the suppression of the growth of cancerous cells or effect the destruction of all or less than all cancerous cells of ductal epithelial origin. In a specific embodiment of the therapeutic methods of the present invention, epithelial cells of the mammary gland are contacted with a cytotoxic cell specific vector so as to destroy cancerous cells of ductal epithelial origin. In another specific embodiment of the therapeutic methods of the present invention, myoepithelial cells of the gland are contacted with a cell specific vector comprising a polynucleotide encoding suppressor molecule so as to inhibit the progression of cancers of ductal epithelial origin. In such therapeutic methods, the epithelial-destroying agent should suppress the growth of or destroy all of the diseased or malignant epithelium. In addition, the ductal epithelium immediately surrounding the diseased/malignant epithelium also preferably should be suppressed or destroyed.

Combined Therapeutic/Prophylactic Methods for Treating a Breast Duct

The present invention also provides methods of treating the ductal epithelium of a mammary gland using prophylactic and therapeutic methods known in the art in combination with the prophylactic and therapeutic methods disclosed herein. Examples of such art accepted methods include surgical removal of the cancerous tissue, radiation therapy and chemotherapy. Such combination methods would then comprise an art accepted methods such as the surgical removal of the cancerous tissue and then further contacting, either concomitantly with or subsequently to the therapeutic treatment, the ductal epithelium of the mammary gland, e.g., by ductal cannulation, with the cell specific vectors disclosed herein, so as to suppress or destroy any remaining cancerous cells and noncancerous cells and to inhibit the spread of cancer.

Diagnostic Methods for Evaluating a Breast Duct for Cancer

The present invention further allows for the utilization of cell specific vectors containing reporter genes in diagnostic studies which can evaluate the expansion of a specific cell lineage. Specifically, using the selective transduction methods described herein, one can determine whether a group of cells (such as proliferating cells) within the duct of a breast belongs to either the epithelial or myoepithelial lineages. For example one can use ductal cannulation to expose luminal epithelial cells with a cell specific vector (such as replication-defective rAd) containing a reporter gene in order to evaluate the expansion of this cancer associated lineage. Alternatively, one can use ductal cannulation to expose myoepithelial cells with a cell specific vector (such as rAAV) containing a reporter gene in order to evaluate the expansion of this lineage. In this way a practitioner can determine whether a group of cells (such as a group of proliferating cells) is, like luminal epithelial cells, prone to cancer. In addition, methods utilizing cell specific vectors containing reporter genes can be used to facilitate the assessment of the presence of occult cancer cells, thereby aiding in long term prognosis and treatment.

In a preferred embodiment of the invention, the method consists of determining the lineage of a cell in a ductal system in a mammary gland selected from the group consisting of a luminal epithelial cell and a myoepithelial cell, by using ductal cannulation to contact the cell with vector containing a reporter gene, wherein the vector selectively targets a molecule that is expressed on the luminal epithelial cell or the myoepithelial cell. As shown for example in FIGS. **3** and **4**, the expression of the reporter gene can be used as an indication of transduction of the cell with the specificity of the vector providing the information as to the cell lineage.

A wide variety of reporter genes and assays that are known in the art can be adapted to the diagnostic methods disclosed herein. For example, a reporter gene can encode an enzyme which produces colorimetric or fluorometric change in the host cell which is detectable by in situ analysis and which is a quantitative or semi-quantitative function of transcriptional activation. Exemplary enzymes include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes capable of being detected by activity which generates a chromophore or fluorophore as will be known to those skilled in the art. A preferred example is *E. coli* beta-galactosidase disclosed herein. This enzyme produces a color change upon cleavage of the indigogenic substrate indolyl-B-D-galactoside by cells bearing beta-galactosidase (see, e.g., Goring et al., Science, 235:456–458 (1987) and Price et al., Proc. Natl. Acad. Sci. U.S.A., 84:156–160 (1987)). This enzyme is preferred because the endogenous β-galactosidase activity in mammalian cells ordinarily is quite low, the analytic screening system using β-galactosidase is not hampered by host cell background.

Compositions of the Invention

In order to facilitate the utilization of vectors useful in gene therapy targeting cells of the breast duct, immortalized myoepithelial cell lines and transplantable xenografts were established from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-X; HMS-3, HMS-3X), breast (HMS-4, HMS-4X) and bronchus (HMS-6, HMS-6X) (M. D. Sternlicht et al. (1996) In Vitro, 32:550563; M. D. Sternlicht et al. (1997) Clin. Cancer Res., 3:1949–1958; Z. Shao et al. (1998) Exper. Cell Res., 241:394–403). These cell lines and xenografts express identical myoepithelial markers as their in situ counterparts and display an essentially normal diploid karyotype. The myoepithelial cell lines and xenografts and myoepithelial cells in situ constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, I-1 antitrypsin, thrombospondin-1, soluble bFGF receptors, and maspin. These suppressor molecules are well known in the art. (See generally, Sternlict et al., Lab. Invest. 74(4) :781–796 (1996) and Sternlict et al., Med. Hypo. 48:37–46 (1997). In addition, for TIMP-1, see U.S. Pat. No. 5,595,885, for protease nexin-II, see U.S. Pat. No. 5,213,962, for α-1 antitrypsin see U.S. Pat. No. 5,736,379, for thrombospondin-1 see U.S. Pat. No. 5,648,461, for bFGF receptors see 5,750,371, and for maspin see U.S. Pat. No. 5,470,970.

The human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial migration and proliferation (angiogenesis) in vitro. The myoepithelial cell lines also inhibit breast carcinoma proliferation through an induction of breast carcinoma cell apoptosis, a phenomenon which occurs at high levels within DCIS (Z. Shao et al. (1998) Exper. Cell Res., 241:394–403). On the basis of immunoprecipitation studies, myoepithelial maspin seems to be the major effector molecule which inhibits invasion and thrombospondin-1 seems to be the major effector molecule which inhibits angiogenesis (S. Bodis et al. (1996) Cancer, 77:1831–1835). Myoepithelial nitric oxide seems to the major effector molecule which inhibits breast carcinoma proliferation through its induction of apoptosis (Z. Shao et al. (1998) Multidiscip. Symposium on Breast Disease).

The present invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1

Treansections of Cells whit Adenovirus

This example relates to the finding is that primary breast ductal epithelial cells express CAR and are easily transduced with rAd whereas myoepithelial cells lack CAR and are completely resistant. CAR is thought to mediate, in part, the attachment of adenovirus to the cell membrane (J. Bergelson et al. (1997) Science 275:1320–1323). In this context, one can use the rAd studies described below as an illustration of methods of the present invention.

rAd Studies: One can use a replication-defective rAd2 (see e.g. Hashimoto et al., Biochem. Biophys. Res. Comm., 240: 88–92 (1997)) with a β-galactosidase reporter and measure its ability to transduce epithelial cells compared to myoepithelial cells through ex vivo and in vivo approaches. In addition, one can use mastectomy specimens and deliver the rAd intraductally as was done with rAAV in Example 2 below. Because rAd does not does not integrate (unlike rAVV), the incubation period will probably be less (on the order of 72–96 hours). One can detect β-galactosidase by a substrate assay in frozen sections of the mastectomy specimens. One can then compare myoepithelial to epithelial colorimetric development. In parallel experiments one can deliver intraductally rAd to anesthetized rabbits and after a few days observe β-galactosidase activity in their breast sections. At this point, one can compare epithelial activity with myoepithelial activity. Even in situations using a replication-defective virus one can nevertheless measure adenovirus titers in the feces and urine of the rabbits by ELISA to evaluate how the myoepithelial layer offers a barrier to systemic infection.

Example 2

Transfection of Cells with Adeno-associated Virus

This example relates to the finding that unlike epithelial cells expression of CAR, myoepithelial cells, instead,

express cell surface heparin sulfate proteoglycan (HS) and are easily transduced with rAAV. As discussed in Example 1 above, primary ductal epithelial cells have absence of this proteoglycan and are resistant to rAAV. Cell surface HS is thought to be a receptor for rAAV which determines transducibility (C. Summerford and R. J. Samulski, J. Virology 72:1438–1445). In this context, one can use the rAAV studies described below as an illustration of methods of the present invention. Up until we established that rAAV can be used to successfully transfect myoepithelial cells (HMS-1), our myoepithelial cell lines were completely resistant to transfection by all conventional means.

rAAV Studies: One can begin with rAAV (see e.g. Flannery et al., P.N.A.S., 94: 6916–6921, (1997)). This recombinant virus contains a CMV promoter and the human green fluorescent protein. One can then inject increasing titers of this virus together with a supravital dye, e.g. lymphazurin (the sentinel node dye) to help guide the injections into the tumor's center. One can then inject the myoepithelial xenografts disclosed herein (HMS-X, HMS-3X, HMS-4X, HMS-6X) of a size of 1 cm in diameter and after 1–3 weeks extirpate these tumors, cut thin frozen sections and study the radial distribution of fluorescence in relation to the azure blue dye. As controls, one can use non-myoepithelial carcinoma tumors where one would expect transduction to be absent or minimal. One can then determine how rAAV transduction into myoepithelial cells followed by recombinant gene expression occurs in vivo by these direct tumoral injection studies. If positive results are obtained one can deliver rAAV intraductally via nipple cannulation into human mastectomy specimens ex vivo and into rabbit nipples in vivo. After 24 hours one can initiate organ culture explants of the mastectomies and then after several weeks observe fluorescence in myoepithelial cells compared to ductal epithelial cell. Since rabbits have eight nipples with 4 ductal systems each they provide a relatively simple animal model to test intraductal gene delivery. After three weeks one can sacrifice the rabbits, section their breasts and compare myoepithelial to epithelial fluorescence.

Example 3

Verifying Observations Concerning Cell-specific Targeting of Recombinant Adenovirus (rAd) and Recombinant Adeno-associated Virus (rAAV) on Epithelial and Myoepithelial Cells Respectively

Using in vitro experiments, we expand upon our earlier findings and outline the parameters that will facilitate in vivo practice of the invention. These parameters include determinations of dose (titer), frequency of administration, period of incubation, period of observation, etc. Controls for all of these correlative in vitro experiments include both non-reporter vector or no vector.

In addition, we rule out the possibility of a pseudotransduction phenomenon occurring in these examples (vector-mediated protein delivery rather than gene expression) especially in the rAAV experiments where this phenomenon has been observed by others. For example, in experiments where successful reporter gene product is observed, we verify that it is gene expression that we are delivering rather than just protein by carrying out a titer dilution experiment. Specifically, the effects of titer dilution should be to decrease protein staining in all cells if we are dealing with pseudotransfection; in true transfection there should be a decrease in the number of cells showing marker protein but the intensity of staining in the positive cells should not decrease. Our results establish that we are dealing with true transfection since we have made the latter observations. Furthermore in our in vitro rAAV experiments performed to date, the intensity of the reporter staining to human green fluorescent protein in the transfected HMS-1 cells increased at 3 weeks verus 1 week after transfection. This increase in reporter protein with increasing passage is indicative of true gene expression because rAAV delivered genes are always a bit slow to show full expression. If we were experiencing pseudotransfection (vector-mediated protein delivery, the reporter staining would decrease with passage.

In addition, we have backed up our proposed marker gene expression assays with a molecular genetic analysis showing the presence of virus by PCR studies.

Expanding upon our initial studies we conducted kinetic studies of transduction efficiency of reporter genes via their respective viral vectors into epithelial and myoepithelial cells in vitro.

In addition, we confirm the usefulness of E1A-deleted rAd containing two different promoters known in the art, i.e., CMV and RSV LTR.

Dose-response studies designed to determine whether there is a relationship between the vector multiplicity of infection (moi) and the efficiency of reporter gene (β-galactosidase) gene transfer into breast epithelial cells (HMEC) are included herein. Specifically, we observe that a moi of 10^2 will achieve 100% transduction efficiency. At the same time we monitored myoepithelial cells for resistance to rAd transduction and determined that their resistance is absolute. For example, they are completely resistant to transfection even with a moi of 10^4.

We also conducted a time course of exposure to rAd over 1–10 hours to determine whether transfection efficiency is enhanced with a longer time of exposure. It is not.

In the case of rAd, we monitored reporter gene expression over time following infection to determine its rate of expression and its decay. We find a half life of expression of 48 hours. We repeated these in vitro studies with rAAV targeting myoepithelial cells. Since rAAV as opposed to rAd integrates into the genome but requires second strand synthesis during cell division before expression occurs (since rAAV is single stranded), we would expect a longer time course after transfection to observe expression but a shallower or no rate of decay. In this context, observe that maximal expression occurs at 3 weeks with negligible decay.

We also compare reporter expression in myoepithelial cells verus epithelial cells. We evaluate replication-competent and replication-enhanced viruses whose production of viral particles can be enhanced by promoter cis/trans interactions and monitor the lysis of epithelial cells and resistance to lysis of myoepithelial cells. Significant lysis is observed visually or by a dye exclusion method (trypan blue) or by a viability monitoring method like MTT. Similar kinetic experiments have been performed. In the case of the replication-enhanced rAd, exogenous agents such as dexamethasone and/or LPS designed to increase transcription from the enhancer/promoter being used (MUC1 and lactoalbumin) in our rAd-transfected breast epithelial cells (HMEC) have been monitored for effects on viral titer and a 10 fold increase has been observed with a 50% increase in cell lysis. Myoepithelial cells remain completely resistant to the effects of this replication-competent lytic virus.

Example 4

Verifying the Feasibility of Transfecting Suppressor Molecules Such as Maspin

We expand upon our in vitro experiments discussed above by using replication-defective rAd and rAAV that contain not reporter genes but native genes such as maspin and conduct similar kinetic studies. Specifically, we repeated all of the above studies that used reporter genes and instead used actual endogenous suppressor genes like maspin to establish that our approach is physiologically feasible.

Maspin Transfection into Mypepithelial Cells via rAAV Vectors

The mechanisms of maspin's effects on invasion and motility inhibition are completely unknown. Maspin was

initially identified by subtractive hybridization and the differential display method to identify candidate tumor suppressor genes that were defective in human breast carcinoma cells. It is interesting that even in those normal breast cell lines which were used to clone maspin and in those normal breast lines where it has been identified it is present only intracellularly and is not secreted. In contrast to epithelial cells, our myoepithelial cell lines do secrete maspin. The presence of secreted maspin in myoepithelial cells compared to normal epithelial cells and our results on maspin's invasion and motility inhibition of breast carcinoma cells support the hypothesis that maspin is acting as a paracrine and not an autocrine tumor suppressor.

Myoepithelial cells seem to have the unique ability to secrete this serpin. Therefore we exploited this property of myoepithelium by transfecting rmaspin into HMS-1 via rAAV in vitro to achieve maspin overexpression. rAAV containing the CMV promoter, for example, was used to modulate a full length maspin cDNA bgated into this vector. Levels of secreted rmaspin were determined by Western blot after several weeks. As shown in FIG. 7, myoepithelial cells overexpressing maspin are more effective at blocking carcinoma cell invasion in MATRIGEL matrix. Such MATRIGEL matrix assays are well known in the art and provide a model for breast cancer systems (see e.g. Bae et al., Breast Cancer Res. Tret. 24(3): 241–55 (1993). Specifically, the effects of transfected myoepithelial clones expressing rmaspin in invasion inhibition assays show a 200% increase in inhibition of invasion. Consequently, these results demonstrate the feasibility of using the overexpression of maspin in myoepithelial as an in vivo gene therapy strategy.

Example 5

In Vivo Studies

The ex vivo findings discussed in Examples 1–4 above illustrate how one can, using an intraductal approach, selectively target and destroy breast epithelium in vivo yet spare the underlying myoepithelium with rAd and/or alternatively selectively target the myoepithelium in vivo with rAAV and bolster its defense with genes such as maspin.

Specifically, using the preliminary ex vivo and in vivo studies disclosed herein, one can selectively target breast myoepithelium and breast epithelium with these vector-specific approaches. Therefore one can proceed in the following manner: use a replication-competent lytic adenovirus or a replication-competent lytic adenovirus containing cis elements which stimulate replication when specific trans factors present in breast epithelium are encountered (a candidate cis element would be the lactoalbumin promoter or the MUCI promoter). One can then evaluate the destruction of breast epithelium at risk for developing cancer and in effect perform a "prophylactic mastectomy" without having to remove the breast. Whether the myoepithelial layer would serve as an effective barrier against systemic infection when a replication-competent lytic adenovirus virus is used can be determined by urine and feces ELISA. The resistance of myoepithelial cells to adenovirus infection makes it likely that this layer offers a defense against systemic infection. Alternatively one can use rAAV to selectively target myoepithelium and deliver to it a candidate gene such as maspin to bolster its defensive abilities.

What is claimed is:

1. A method of selectively transducing a cell within a mixed population of ductal epithelial and myoepithelial cells, comprising the step of contacting the cell with a cell specific vector that transduces the cell through a coxsackie-adenovirus receptor molecule expressed by the ductal epithelial cell or a heparin sulfate proteoglycan molecule expressed by the myoepithelial cell, wherein the cell is a myoepithelial cell.

2. The method according to claim **1** wherein the vector is a recombinant adeno-associated virus.

3. The method according to claim **1**, wherein the vector comprises a gene encoding a polypeptide which inhibits the proliferation of cells.

4. The method according to claim **3**, wherein a control sequence in the recombinant adeno-associated virus contains a cis element which modulates the expression of the gene in the presence of a trans factor present in the myoepithelial cell.

5. The method according to claim **3**, wherein the polypeptide inhibits the proliferation of a ductal epithelial cell.

6. The method according to claim **3**, wherein the polypeptide inhibits the invasion of a ductal epithelial cell.

7. The method according to claim **3**, wherein the polypeptide inhibits endothelial migration.

8. The method according to claim **3**, wherein the polypeptide inhibits angiogenesis.

9. The method according to claim **3**, wherein the polypeptide increases the production of nitric oxide by the myoepithelial cell.

10. The method according to claim **3**, wherein the polypeptide induces apoptosis in a ductal epithelial cell.

11. The method according to claim **3**, wherein the polypeptide is selected from the group consisting of maspin, thrombospondin-1, TIMP-1, protease nexin-II, α-1 antitrypsin and soluble bFGF receptor.

12. A method of selectively transducing a myoepithelial cell within a mixed population of ductal epithelial and myoepithelial cells in a ductal system in a mammary gland, comprising the step of contacting, by ductal cannulation, the cell with a vector that targets a heparin sulfate proteoglycan molecule expressed by the cell.

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